EFFECT OF VARIOUS AGENTS ON THE BLOOD FLOW THROUGH THE CORONARY ARTERIES AND VEINS.[1]

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PLATES XLVII–LI.

Porter sums up the history of the early work on the coronary circulation as follows:

In 1689, J. Baptiste Scaramenci demonstrated that the vessels were squeezed during systole and filled in diastole. This view was supported by Stroem, in 1707, with the fact that the aortic valves close the coronary openings during systole. In 1855, Hyrtl stated that blood spurted from a cut coronary during systole, and his findings were confirmed by Perls, although they failed to mention which end of the vessel spurted. Klug, in 1876, ligated the vessels in diastole and systole and then coagulated the blood. He found a greater quantity present during systole.

This earlier work was followed by that of Chaveau and Rebatel who recorded the velocity of flow. They determined that there were two fillings, one in systole and one in diastole. Martin and Sedgwick, in 1895, showed that the pulse wave in the coronaries was synchronous with that in the carotid arteries, although they did not deny the possibility of a diastolic filling. In the same year, Martin observed the vagus control of vasodilation in the cardiac vessels.

With the introduction of perfusion by Martin, in 1890, there began a great deal of work concerning the effect of various factors on the coronary flow. Magrath and Kennedy, in 1897, demonstrated that the strength and not the frequency of the heart was dependent on the amount of coronary circulation. In the following year, Porter demonstrated that the flow is influenced by both the rate and strength of the heart beat. I. Hyde showed that distention of the ventricle diminished coronary flow. Pratt pointed

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out that the circulation was not entirely due to the coronary arteries, but was probably in part from the veins of Thebesius and retrograde flow in the coronary veins. Paul Maass established the nervous control of the coronary vasomotors, and showed that the dilators run in the sympathetic and the constrictors, in the vagus. Hedbom, in 1899, concluded that caffeine, atropin, and quinine act as dilators of the coronary vessels.

In 1903, Francois-Franck used the method of instantaneous photography of the superficial arteries of the heart. With amyl nitrite he found a marked vasodilatation.

O. Loeb in the same year worked out the effect of several drugs on the coronary arteries of the perfused heart as follows: strophanthus caused no change in coronaries or in flow; digotoxin gave a decrease in flow, and, therefore, must cause constriction of the coronaries; caffeine, theobromine, and diuretin caused no marked increase in flow; amyl nitrite in small doses had very little effect on the size of the coronary vessels.

Langendorff, in 1906, found that adrenalin gave only an uncertain effect on the coronaries in the perfused heart, while, in the following year, he demonstrated upon an excised strip of a large coronary artery that it produced, if anything, some dilatation of the vessel.

Wiggers, in a paper published in 1909, demonstrated by a special method of perfusion that adrenalin constricted the coronary vessels. By recording the outflow of blood from a wounded coronary vein of the living heart *in situ,* he also showed that stimulation of the vagus caused a diminished flow, and reasoned from this observation that diminished flow must be due to vasoconstriction.

It can be seen in this previous work that there is some confusion as to the results of various drugs. It is also probable that in the heart under normal condition, in contradistinction to the perfused heart, there are factors other than those affecting the heart which might be influenced by the drug. These in turn would affect the coronary flow. For this reason the results obtained with perfused hearts are not exactly comparable to those due to the action of the drug upon the blood supply of the heart *in situ.*

Francois-Franck's work was carried out under as nearly normal

conditions as possible, but his photographs show only the changes in the superficial vessels and do not deal with the blood supply of the heart muscle.

The experiments embodied in the present paper were begun at the suggestion and under the direction of Dr. A. D. Hirschfelder, in 1909, quite independently of the work of Wiggers and before his paper on coronary circulation was announced. The method employed was substantially the same as that which Wiggers had used for showing the circulation through the pulmonary artery. In this way any change in blood supply to the heart muscle could be demonstrated without regard to the various factors which might enter into its production. The experiments were performed on dogs, because their coronary veins are of sufficient size to be punctured readily and permit a slow dropping of blood from the wound.

The following procedure was adopted. The animal was anesthetized and kept alive by artificial respiration. Cutting away the chest wall as freely as possible exposed the heart in such a manner that there was no interference with the falling drops from the coronary vein. To record venous pressure a cannula was placed in the right auricle. The carotid pressure was noted by a Hürthle recording manometer. The animal was suspended face downward and the heart kept from dropping forward by fastening the apex to the base of the diaphragm with a long ligature. It was now necessary to make a small wound in one of the veins following the ramus descendens of the left branch of the coronary artery. The blood was allowed to drop from this opening upon a small mica plate fastened at an angle of 45° to a long lever of straw. The fulcrum of this lever rested on a tambour, so that each drop caused a movement of a recording lever.

Magrath and Kennedy have shown that the size of drops does not vary with differences in rapidity of flow or changes in strength or rate of the heart's contraction. Thus the number of drops furnishes a quantitative estimate of the flow.

There is no doubt that there is considerable shock entailed in the operation, but Wiggers has shown that the vasomotor action is still present in the coronaries after the same operation. We can assume, therefore, that it is still present in these experiments.

Pratt holds that some of the coronary circulation is a retrograde flow in the coronary veins. As we recorded the changes of pressure in the right auricle, we did not deem it necessary to ligate the vein above the point of injury.

At the beginning of these experiments, much trouble was caused by clotting in the punctured veins. To obviate this difficulty, the coagulability of the blood was previously reduced. Slowly withdrawing 250 to 300 cubic centimeters of blood, normal salt solution was simultaneously run in the femoral vein to an excess of 100 cubic centimeters. The latter compensated for the loss of blood during the operation. In some instances it was found necessary to reopen the vein occasionally. Periodical perfusions to replace the blood lost continuously at the coronary vein during experimentation soon eliminated entirely the factor of clotting. In practically no instance was any untoward result to the heart produced by the procedure, while in many instances the heart was greatly benefited, probably by the warmth and increase in quantity of circulating fluid.

All the drugs were given by hypodermic injection into the femoral vein. The order in which they were administered was constantly changed to rule out the factors of loss of vasomotor tone and the residue effect of other drugs. Normal tracings were taken before each drug for comparison. At the beginning of the work, those drugs were first chosen which were known to have the greatest effect on the vasomotor system. Adrenalin was used to represent the vasoconstrictor element, and for vasodilatation amyl nitrite and nitroglycerin were used.

Adrenalin.—The effects previously obtained with this drug have been somewhat contradictory. Schäfer could not produce any constriction, while Langendorff was even able to obtain a dilatation. Wiggers, on the contrary, demonstrated a marked vasoconstriction in an inactive heart.

In these experiments a fresh solution of 1 to 1,000 supradrenalin was used, doses varying from three to six drops being sufficient to produce a pronounced effect on the peripheral circulation. Twenty-five injections of this drug gave, on the whole, very uniform results. A typical chart is shown in Plate XLVII, Fig. 1 and Fig. 1 in text.

Following the introduction of the drug the marked effect on the peripheral circulation can be noted. A general increase in blood pressure with a primary decrease in cardiac output is followed shortly by increased strength of heart contraction and greater systolic output. The heart rate is, as a rule, only slightly affected. It

is slowed during the height of the effect of the drug, but may become more rapid as the blood pressure returns to its normal level. Simultaneous with the rise of arterial pressure, the flow through the coronary arteries, as judged by the number of drops, increases in volume. This increase follows the curve of arterial pressure, and

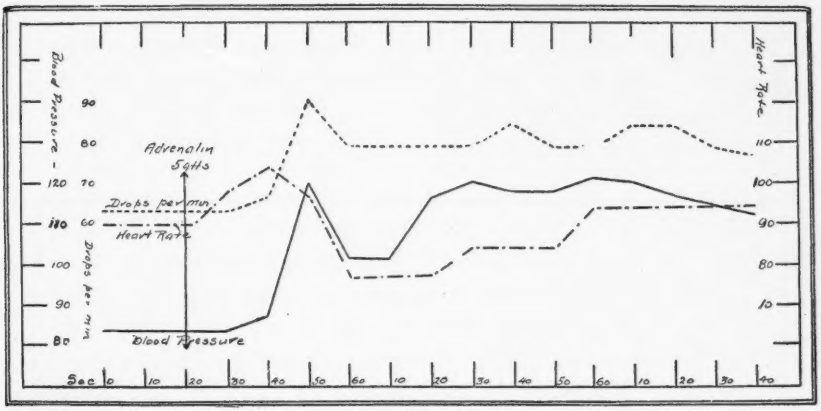


FIG. 1.

decreases gradually with its return to normal. In a few cases, simultaneously with the primary rise of blood pressure, there occurred a transitory slowing of the coronary flow, followed by a sudden increase. It might appear that in these instances the coronary arteries had entered into the general vasoconstriction. The number of such results was so few, as compared to those of opposite effect, that they hardly permit of any deduction.

The pressure as recorded in the right auricle usually showed a slight elevation, but this elevation did not begin until the blood pressure and flow had already reached their maximum. The increased outflow from the vein, therefore, cannot be attributed to a backflow from the veins, but must be considered as arterial in origin.

In the heart itself several things were noted which substantiate the graphic results. The organ changed to a much brighter hue and its contractions became stronger. The blood flowing from the wound, which had been dark, now changed to a bright crimson.

The flow increased in some cases to an almost constant stream, spurting with each ventricular systole.

From the results obtained, it can hardly be stated just what part vasomotor factor plays during the effect of adrenalin. It is evident, however, that either vasodilatation or constriction is of minor importance. The main element which here governs the blood supply to the heart is the condition of the peripheral blood pressure.

Adrenalin, from prolonged usage, has been shown to cause arterio-sclerosis and myocarditis. The deduction has been that the vasoconstriction of the coronaries caused an insufficient nutrition for the heart. These findings strongly oppose this view, because,

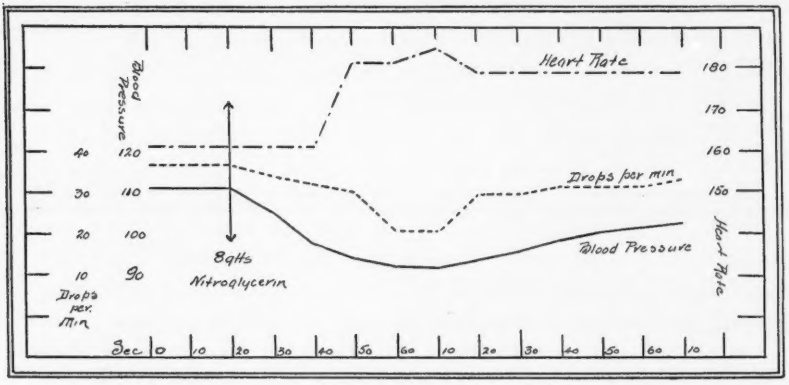


FIG. 2.

even with a certain amount of arterio-sclerosis, the increased blood pressure from the adrenalin should improve the nutrition of the heart muscle. The possibility suggests itself, however, that the resultant changes may be due to the increased circulation of the toxic product.

Nitroglycerin.—Fifteen experiments were performed with this drug. Injections were made slowly into the vein, and were continued in each case until after the administration of a sufficient dose there was a fall in peripheral blood pressure (Plate XLVIII, Fig. 2 and Fig. 2 in text). This decrease is greater in the minimal than in the maximal, so that the pulse pressure is increased through-

out. The frequency and strength of the heart's contractions remained practically the same during the experiment.

Coincident with this fall of blood pressure, the flow from the coronary vessels is diminished in amount and velocity. The latter follows a course quite parallel to the blood pressure curve, and both gradually return to their former height.

Although there was apparently some dilatation of the right side of the heart, there was little change in the pressure in the right auricle. Therefore, in this case a retrograde flow in the coronary veins need not be considered. In some instances where cessation of the heart beat took place, the venous pressure was markedly increased, but this increase never produced any effect on the coronary flow. The latter always diminished according to the decline of the curve of arterial pressure.

Amyl Nitrite.—The result of ten experiments with this drug show practically the same conditions present as with nitroglycerin. The effect, however, was somewhat more transient. We have decrease in the blood pressure with simultaneous slowing of the coronary circulation (Plate XLIX, Fig. 3 and Fig. 3 in text).

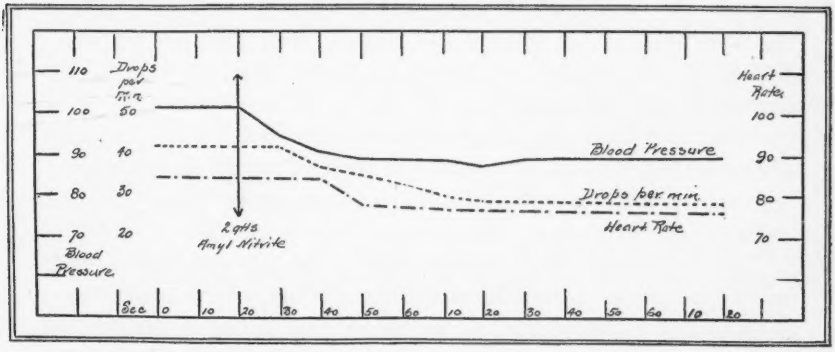


FIG. 3.

Where no other factors entered, in no instance was the retardation of the flow proportional to that obtained with nitroglycerin. This permits of two explanations. The toxicity of the drug was so great that only slight effects could be produced without injury

to the heart. Some effects, however, were comparable to those of nitroglycerin, and even here the change is not as marked. It is possible that with this drug there is present some vasodilation of the coronary vessels. This has been the common belief, though O. Loeb produced the opposite effect. The vasodilation, if present, was, however, not sufficient to overcome the results of the lowered peripheral pressure, and the blood supply to the heart was diminished.

The therapeutic value of amyl nitrite, in view of these findings, cannot be explained by the increased nutrition of the heart, as it has been generally supposed. It would be impossible to say without experimental evidence how great would be its effect on conditions of spasm of the coronary arteries. We can assume, however, from our results that considerable vasodilation would be necessary to counterbalance the effect of the lowered blood pressure.

Osler states that in anginal attacks there is usually a peripheral vasoconstriction and increased arterial tension. It would appear, therefore, that a large amount of the relief is probably obtained through the lowering of the general blood pressure and consequent lessened heart work.

Transfusion with Normal Salt Solution.—To obtain the effect of a simple increase in blood pressure, the flow was recorded during the slow injection of normal salt solution into the femoral vein. This produced a gradual rise in blood pressure, which, owing to the increased rate and strength of the heart, was manifested more in the arterial than in the venous system. As shown in the tracing (Plate L, Fig. 4 and Fig. 4 in text), there is a marked increase in coronary circulation. It begins at the initial rise of blood pressure and gradually increases as the pressure curve ascends.

The mechanical effect of the dilution of the circulating fluid might be given as a partial cause for the effect. It would operate in two ways: either causing an actual increase in the circulation, or an apparent one due to the change in size of the drops. It must be remembered, however, that the viscosity of the blood has already been lowered to about the consistency of salt solution to prevent clotting. The small change due to the added salt solution would, therefore, hardly show its effect.

Inhalation of Tobacco Smoke.—It has been the general belief

that nicotine as inhaled in tobacco smoke causes a constriction of the coronary arteries. This would decrease the blood supplied to the heart muscle. Lee devised a method by which tobacco smoke could be introduced by means of artificial respiration. With this method he demonstrated that its administration caused a rise in the peripheral blood pressure. This observation led us to believe that

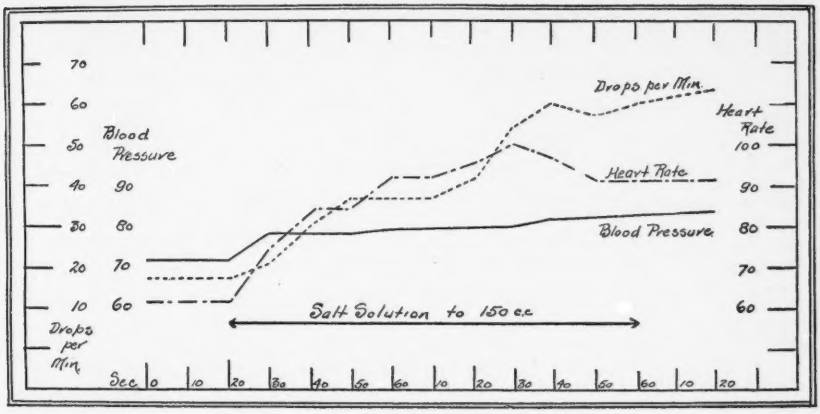


FIG. 4.

the blood supply is probably increased, and such proved to be the case in the few experiments we attempted (Plate LI, Fig. 5). Coupled with the rise of arterial pressure, the coronary flow showed a proportional increase. If there is any vasoconstriction, it is not sufficient to compensate for the results of the elevated arterial pressure. The blood supplied to the heart is, therefore, augmented by the inhalation of tobacco smoke rather than decreased.

Cardiac Stimulants.—Several other drugs that are supposed to have more or less of a selective action on the coronary vessels have been tried: digitalis, strophanthus, caffeine, strychnine, and theobromine in the form of agurin. After administration of all of these, however, we were unable to demonstrate any variations in the outflow from the coronary veins.

There are many factors which influence the coronary circulation. Magrath and Kennedy, as well as Porter, demonstrated that the

strength of the heart's contraction had its effect. Porter also showed that the flow was modified by the heart rate. In my experiments the strength rather than the rate seemed to produce the most marked results. Even this statement, however, has not an absolute foundation, because the increased strength produces a rise of blood pressure which may have been the cause of the variation in coronary flow.

It has been definitely shown that there is a vasomotor action in the coronary vessels. The influence of this action is probably present in all of the experiments, but appears most definitely in those with amyl nitrite.

These three factors are of importance, and under certain conditions probably exert a marked influence. But the main element which controls the coronary circulation is the arterial blood pressure. Its effects are sufficient to overbalance all the other influences. Thus the blood supply of the heart uniformly depends upon the pressure at the openings of the coronary vessels in the aorta.

Another phase is that shown by Langendorff, Paul Maass, and Magrath and Kennedy, namely, that both the rate and strength of the heart's contraction are dependent on the velocity of flow in the coronaries. This fact is very well shown in the results of both adrenalin and salt solution. After the increased flow is inaugurated, both the heart rate and strength are increased.

SUMMARY.

The results obtained show that adrenalin, transfusion with salt solution, and the inhalation of tobacco smoke caused an increased circulation in the coronary vessels. Amyl nitrite and nitroglycerin produce the opposite effect. Digitalis, strophanthus, caffeine, and theobromine give no change in the velocity of circulation.

We can conclude from the results that the blood pressure is the main element which influences coronary circulation, while the other factors play only a minor part in its variations.

In conclusion I wish to express my thanks to Dr. L. F. Barker for permission to work in his clinic, to Dr. A. D. Hirschfelder for his aid and suggestions, and to Dr. Elizabeth S. Hellweg for her valuable assistance in the experiments.

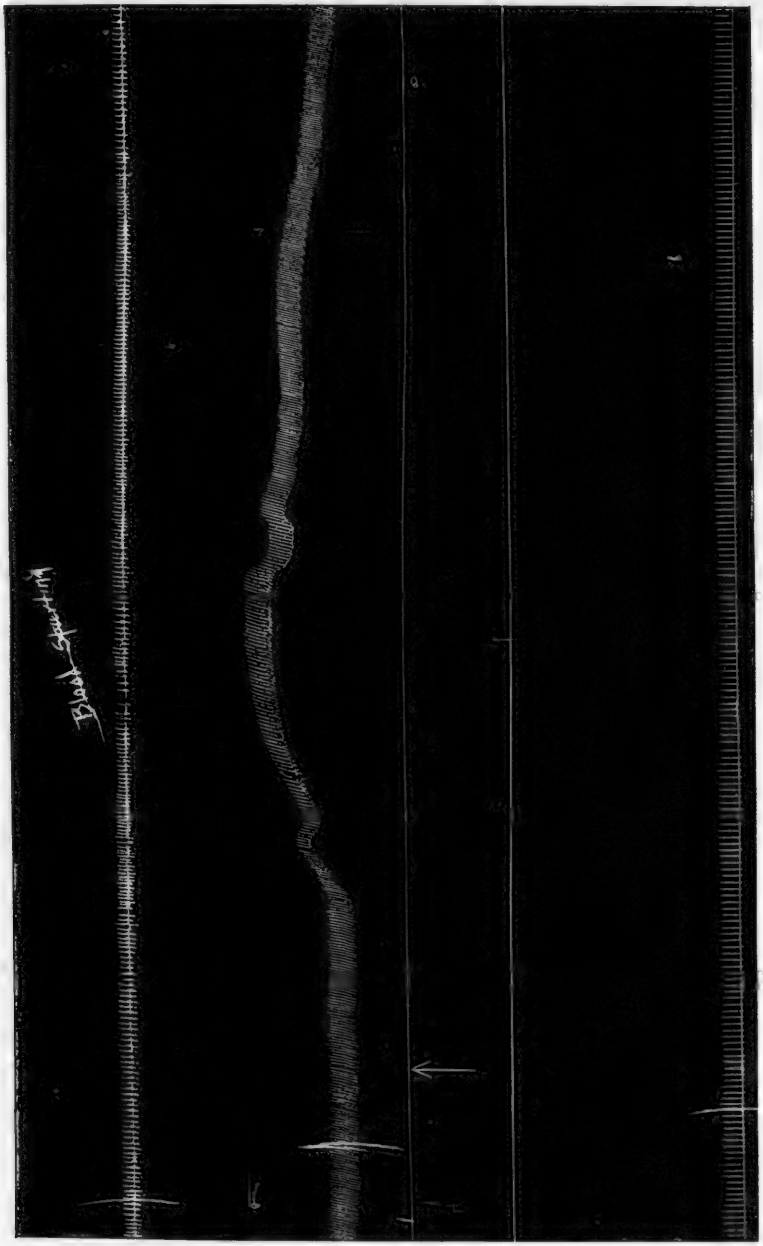


FIG. 1

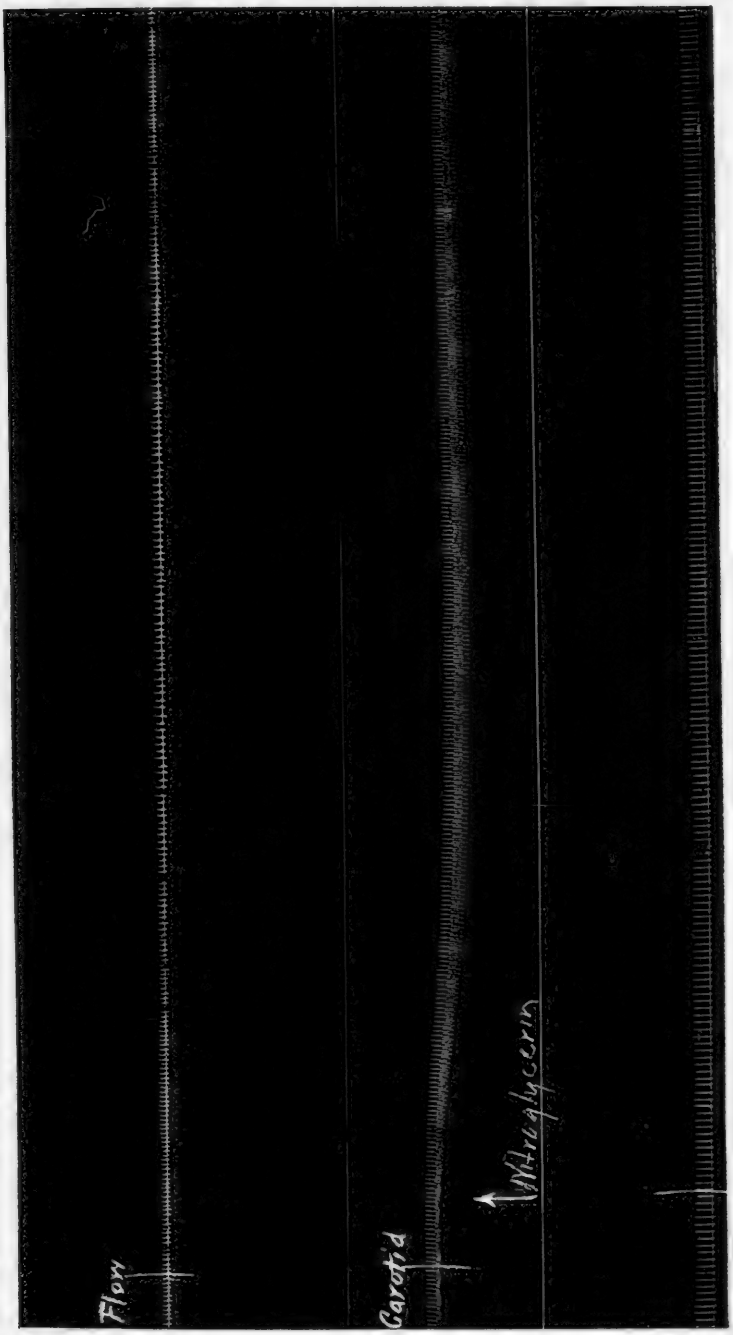


FIG. 2.

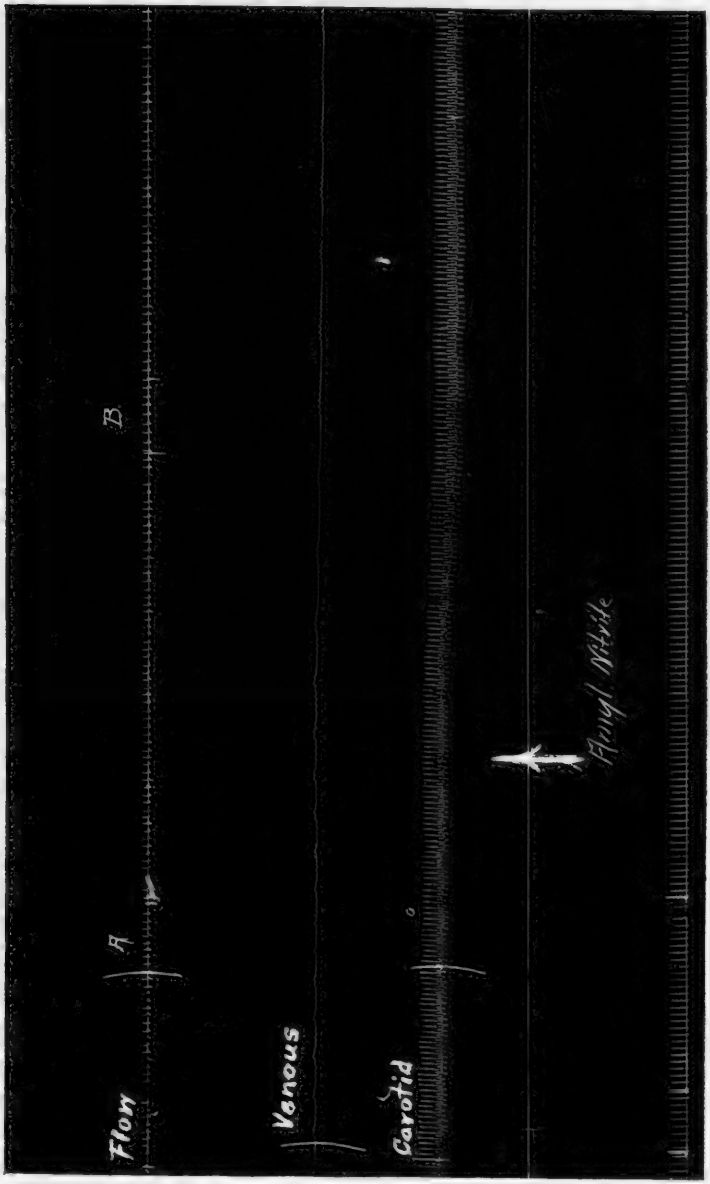


FIG. 3.

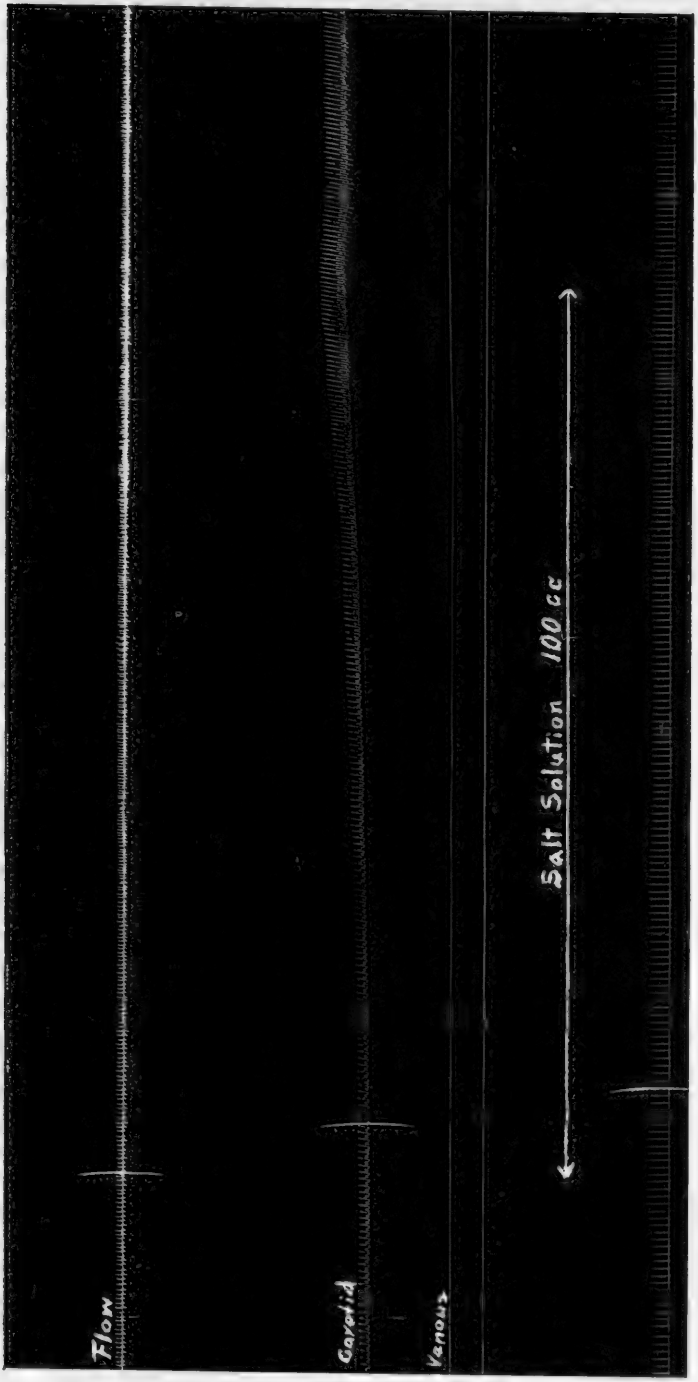
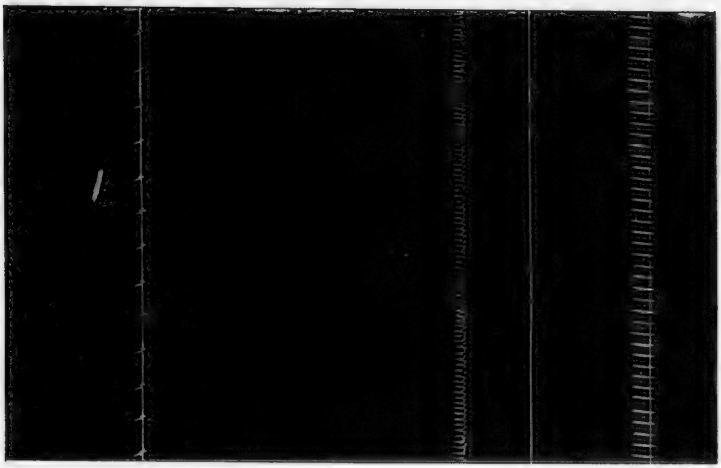


Fig. 4.

During inhalation.



Before inhalation.

FIG. 5.

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EXPLANATION OF PLATES

Plate XLVII.

Fig. 1. The curves reading from above downward are: velocity of flow in drops, carotid blood pressure, pressure in right auricle. Base line represents time marked in seconds. Adrenalin injected at arrow.

Plate XLVIII.

Fig. 2. The curves reading from above downward are: coronary flow in drops, pressure in right auricle, carotid pressure (Hürthle). Base line represents time marked in seconds. Nitroglycerin given at arrow.

Plate XLIX.

Fig. 3. The curves reading from above downward are: coronary flow in drops, venous pressure, carotid pressure (Hürthle). Base line represents time marked in seconds. Amyl nitrite given at arrow. Flow at A, 59 drops per minute; at B, 48 drops per minute.

Plate L.

Fig. 4. The curves reading from above downward are: coronary flow in drops, arterial pressure (Hürthle), venous pressure. Base line represents time marked in seconds. Arrow indicates the length of time consumed in the injection.

Plate LI.

Fig. 5. Effect of inhaling tobacco smoke. The curves reading from above downward are: coronary flow in drops, arterial pressure (Hürthle). Base line represents time marked in seconds. The increase in arterial pressure is shown by the height above the base line. The coronary flow shows a marked increase.

ANOTHER POINT OF RESEMBLANCE BETWEEN ANAPHYLACTIC INTOXICATION AND POISONING WITH WITTE'S PEPTON.[1]

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PLATE LII.

Kraus and Biedl (1) have recently called attention to the similarity which exists between the effects of anaphylactic intoxication and poisoning with Witte's pepton. In both conditions there are produced fall in blood pressure from vascular dilatation, leucopenia, and, in meat-fed dogs, loss of coagulability of the blood. Still more striking is the fact that if the Witte's pepton be injected slowly or in small doses the animal becomes immune to a subsequent injection for about twenty-four hours.

W. H. Thompson (2) has shown that the portion of the pepton which acts upon the circulation is not the purified pepton (amphopepton) nor even the secondary or simpler proteoses (deuteroproteoses), but only the higher proteoses (proto- and heteroproteoses) which still contain most of the proteid components (Bausteine) and maintain much of the original architecture of the proteid molecule. Rosenau and Anderson (3) and Wells (4) have shown that one injection of these proteoses does not sensitize to a subsequent injection. On the other hand, Vaughan and Wheeler (5) have been able to split the proteid molecule by boiling with 2 per cent. sodium hydrate in 95 per cent. alcohol, obtaining a toxic portion and a portion which sensitizes to subsequent injection but which is itself not specially toxic. Abderhalden and his pupils (6) have shown that after intravenous injection of horse serum, egg albumin, gliadin, casein, edestin, or gelatin into rabbits, there appears in the serum a proteolytic enzyme which splits the pepton obtained from

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silk (Seidenpepton) and which is not normally present, but injection of any of these proteids seems to cause the appearance of the same enzyme. The effect does not appear to be specific for any one proteid. Many of the above facts, however, as stated by Nicolle (7) suggest a relation between anaphylactic intoxication and hydrolytic cleavage of the proteid molecule.

Accordingly it is of interest to learn all the points of resemblance between the symptomatology of anaphylaxis and those of pepton poisoning. Auer and Lewis (8) have recently called attention to a new phenomenon regularly associated with anaphylactic poisoning, namely, the fact that anaphylactic death in the guinea pig is due to asphyxia from bronchoconstriction. The muscles of the smaller bronchi contract so that they inhibit both the intake and output of air; so that, in spite of tremendous respiratory efforts accompanied by a high degree of negative pressure in the thoracic cavity, the respiratory interchange ceases, air neither enters nor leaves the bronchi, the air imprisoned within the air cells of the lungs becomes overcharged with carbon dioxide, and death from asphyxia results. At autopsy the lungs are found to be much inflated and rigid. They do not collapse, even when cut through. The surface is usually dry, but may be more or less moist in the areas of pulmonary edema which are frequently present. As pointed out by Auer and Lewis, this pulmonary rigidity is a rather unique phenomenon in animal experimentation. It is not brought about by pulmonary edema, as von Basch (9) and his pupils had claimed; for von Basch's theory was based largely upon Grossmann's (10) experiments with muscarin, a drug which, as Dixon and Brodie (11) have shown, itself acts upon the walls of the bronchi to produce bronchoconstriction. Moreover, W. H. Schultz (12) has demonstrated that in anaphylaxis the smooth muscle of the intestine as well as that in the lungs and elsewhere in the body is sensitized to the action of the proteid, so that the contraction of these fibers may be brought about in excised strips upon contact with the proteid.

In a number of experiments in my series in which pulmonary edema of extreme grade was produced either by infusion of fluids, by asphyxia, or by overdose of ether, it was noticed that the lungs

collapsed completely and presented no similarity at all to the lungs found in acute anaphylaxis or in pepton poisoning.

The present series of experiments was carried out upon guinea pigs with the intention of determining whether the bronchoconstriction with pulmonary rigidity described by Auer and Lewis was also present in guinea pigs killed by injection of Witte's pepton. The animals were subjected to a light etherization, the jugular veins dissected out, and the fluid either injected into the vein directly with a large syringe or else a canula was inserted and the fluid allowed to flow in from a burette. In most of the experiments a canula was inserted into the trachea so that the short series of respirations might be recorded from time to time by connecting it up with a large tambour. The number of respirations thus recorded was too short to affect the character of the respiration.

It was found that in all cases in which a fatal dose of Witte's pepton was injected rapidly, the phenomena were similar to those described by Auer and Lewis. Within one or two minutes after the injection of a five cubic centimeter dose, the respiratory movements increased and became mainly abdominal, while the respiratory intake diminished (Plate LII, Fig. 1). During the next couple of minutes breathing became slower and more violent. The accessory muscles were brought into play. In spite of the most violent respiratory effort, no air whatever entered the lungs during the last few gasps, and the animal died from asphyxia (Plate LII, Fig. 2). In death from ordinary asphyxiation (suffocation), on the other hand, air entered the lungs as long as respiratory movements were kept up (Plate LII, Fig. 3).

Upon opening the chest the heart was usually found still beating and was usually not much dilated.

The lungs, in all instances of rapid pepton injection, exactly resembled those described in anaphylactic death by Auer and Lewis. They did not collapse but were sufficiently rigid to form a mold of the chest. Indeed, when cut out of the chest, the lungs supported the heart and kept it raised well off the table. The cut surface of the lung was usually dry, though occasionally slightly edematous, and pieces of these lungs floated upon water, often leaving more than half the tissue above the surface. These effects were not

modified by the administration of atropin in three animals. This condition was in sharp contrast to the control animals killed by asphyxia, ether, etc., in which pulmonary edema was the most striking feature. The lungs of this group of animals collapsed completely, disproving the view of von Basch and his pupils that the edema alone can bring about rigidity of the lungs in broken cardiac compensation. Sections of the edematous lungs, of course, were almost entirely submerged when placed in water.

Tabulation of Experiments.

Date.	Weight in grams.	Injection.	State of lungs.
Jan. 6	250	7 c.c. 10 per cent. pepton intravenously.	Lungs do not collapse. They are rigid and support heart. Cut surface dry.
Jan. 18	420	8 c.c. 10 per cent. pepton intravenously.	Lungs do not collapse. They are rigid and support heart. Cut surface dry.
Jan. 27	510	5 c.c. 10 per cent. pepton intravenously.	Lungs do not collapse. They are rigid and support heart. Cut surface dry Considerable edema of lungs also.
Mar. 30	480	1 mg. atropin, then 10 c.c. 10 per cent. pepton.	Lungs do not collapse. No edema.
Mar. 30	420	2 mg. atropin, then 5 c.c. pepton.	Lungs do not collapse. No edema.
Apr. 29	420	1 mg. atropin, then 3.5 c.c. 10 per cent. pepton.	Lungs do not collapse. Slight edema
Mar. 9	390	20 c.c. 10 per cent. pepton into peritoneal cavity, 5 c.c. intravenously.	Lungs collapsed. No edema.
Apr. 28	390	12.7 c.c. pepton in 34 minutes.	Lungs completely collapsed.
Jan. 20		1 mg. atropin, 26 c.c. 10 per cent. pepton injected slowly into vein. A considerable portion escaped into tissues.	Animal killed by opening the thorax. Heart greatly dilated. Lungs are extremely edematous but collapse completely.
Jan. 7	450	14.5 c.c. 10 per cent. pepton into the thoracic cavity.	No effect. Animal subsequently killed by asphyxiation. Lungs collapsed.
Feb. 2	450	55 c.c. 10 per cent. pepton into peritoneal cavity.	No effect. Animal subsequently killed by asphyxiation. Pleuritic effusion ascites, wet collapsed lungs.
Feb. 22	660	Death from hemorrhage.	Lungs completely collapsed.
Feb. 31	360	Asphyxiated.	Wet collapsed lungs.
Jan. 28	420	Atropin 1 mg., then asphyxiated.	Lungs completely collapsed.
Apr. 28	450	Atropin 1 mg., then killed with ether.	Lungs completely collapsed.

When the pepton solution was injected into the peritoneal cavity, or very slowly into the jugular vein, death occurred much more slowly, and though preceded by gradually increasing respiratory distress it was not accompanied by actual rigidity of the lungs, and the latter collapsed after the chest was opened.

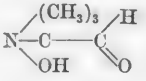
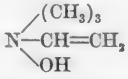
In two rabbits killed by injection of Witte's pepton the lungs collapsed.

The experiments recorded above seem to warrant the addition of pulmonary rigidity and bronchoconstriction to the category of symptoms which express a certain parallelism between the anaphylactic and the pepton poisoning. The amount of proteose necessary to kill a normal guinea pig is, however, almost 500,000 times greater than that of the native proteid which is fatal to a sensitized animal. It seems scarcely likely that the toxic effect of the latter should act through some hydrolytic cleavage products of the proteid molecule, as claimed by Nicolle, though it is possible that within the body the cleavage may occur in a different part of the proteid molecule from that at which it occurs in proteid digestion. Abderhalden and Koelker (13) have reported such differences between the action of yeast enzyme and trypsin upon the polypeptid l-leucyl-glycyl-d-alanin, in which the former splits off the leucin, and the latter the alanin.

Against this view is the fact that none of the products of proteid hydrolysis and none of the polypeptids and none of the aminoacids thus far studied (with the exception of the substances described by Vaughan and Wheeler) have been highly toxic, and even the toxic fraction of the proteid molecule extracted by Vaughan and Wheeler after hydrolysis in alcohol exerts much less effect upon the normal animal than does the specific proteid upon the sensitized. The discrepancy between the huge doses of pepton and the infinitesimal doses of native proteid, far from supporting the hydrolytic theory, renders it conceivable that some of the pepton might be changed into higher proteids by a reversal of the hydrolytic process, *i. e.*, a hydrosynthesis, similar to the hydrosynthesis, which results in the formation of plasteins. It is quite as possible that such higher peptids rather than the lower ones might furnish the material from which the toxic substance is directly formed. It must be remembered that the

peptons of the culture media are used by the bacteria in the synthesis of the ptomaines and the toxins, and it is possible that the reactions within the mammalian organism may be somewhat similar. The fact that, according to Thompson, it is only the higher proteoses, and not the lower ones, which produce the pepton effect seems evidence against the theory of further hydrolysis.

Nolf (14) has shown that the toxic action of pepton is due to the formation of some substance in the liver, and the recent experiments of Manwaring (15) furnish some evidence that the same organ plays a rôle in the development of the anaphylactic reaction.

In this connection it must be borne in mind that hydrolyses and hydrosyntheses are not the only processes which go on in the liver. Dehydrations and desamidations and recombinations with ammonia are no less important. It is a striking fact that certain diamines (ptomaines) and certain betain derivatives (muscarin, ) and neurin,  are among the most poisonous substances known and are produced in the putrefaction of proteids. The toxicity of these substances to normal animals approaches that of the native proteid for the sensitized animal. Moreover, Dixon and Brodie have shown that muscarin and neurin are among the few substances which exert a marked action upon the smooth muscle of the bronchi. This action has a considerable similarity to the bronchoconstriction observed by Auer and Lewis in anaphylaxis and also to that which resulted from the injection of Witte's pepton in the present series of experiments.[2] But though it might be alluring to theorize upon the possibility that a common radical might be responsible for the similar effects in all these instances, the matter is a subject for further experiment rather than for speculation.

[2] It is interesting to note that Sybil May Wheeler, approaching the question of proteid intoxication from an entirely different viewpoint, has noted other points of resemblance between the proteid intoxication and that with the betain derivatives. Moreover, Reid Hunt and Taveau have found that certain synthetic choline derivatives have a toxicity far greater than that of muscarin or neurin, and almost equal to the toxicity of the native proteid for the sensitized animal.

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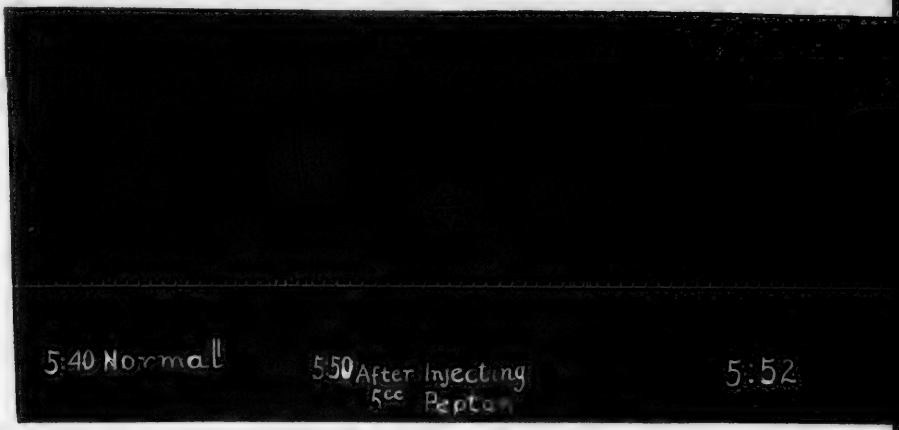
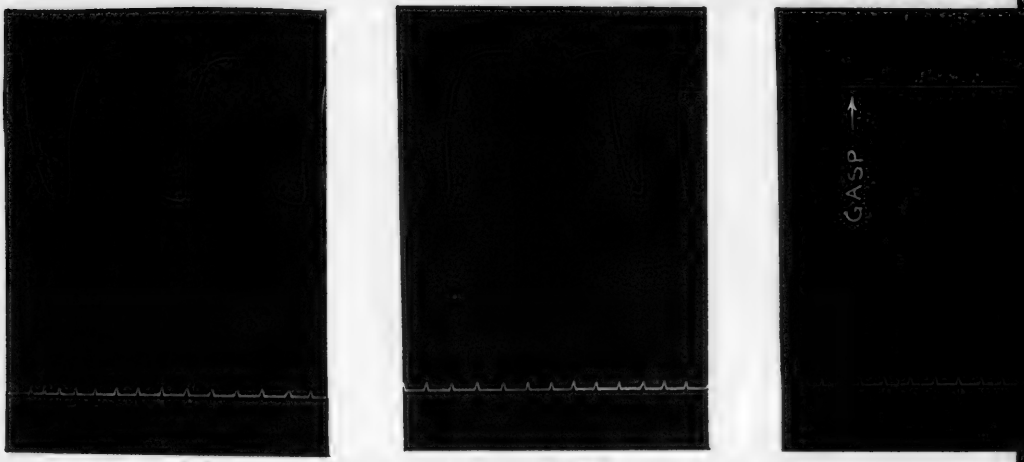
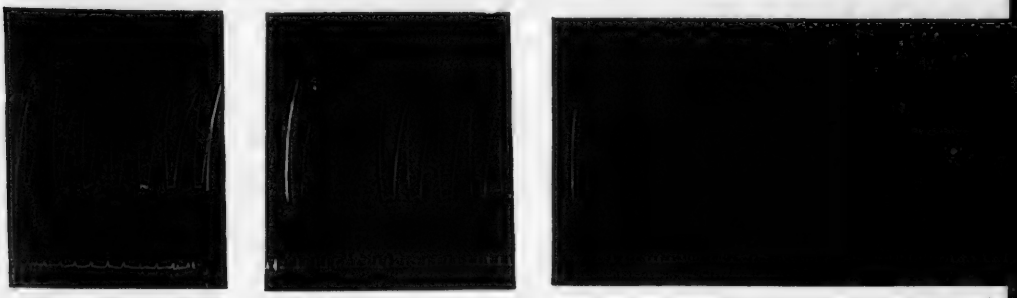


FIG. 1.



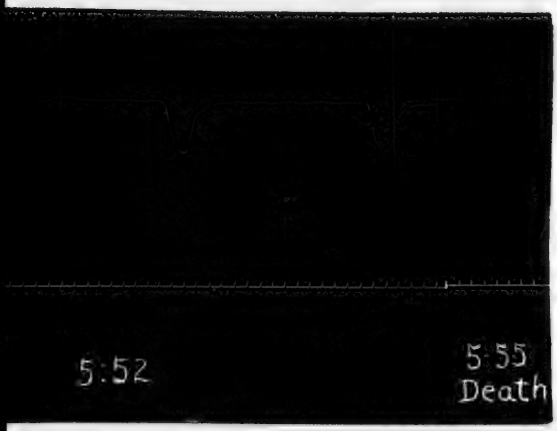
4:00 — Normal. 4:10 — After 1 mg. atropin.

FIG. 2.

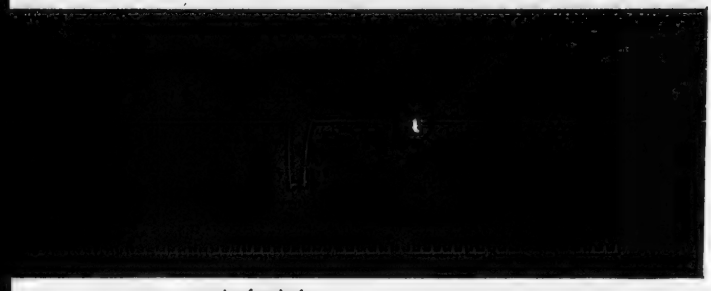


12:00 — Normal. 1:18 — Beginning asphyxiation. 1:22 — As

FIG. 3.



4:16 — After 5 c.c. pepton.



1:22 — Asphyxiation.

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EXPLANATION OF PLATE LII.

FIG. 1. Record taken with tambour attached to tracheal canula showing the diminution and slowing of the respiratory intake just before death from injection of Witte's pepton.

FIG. 2. Record showing that no air entered the lungs during the last few inspiratory gasps after poisioning with pepton.

FIG. 3. Record showing how air enters the guinea pig's lungs during the last few gasps after simple asphyxiation.

STUDIES ON IMMUNITY IN CANCERS OF THE WHITE RAT.[1]

By ISAAC LEVIN.

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The most important biological phenomenon observed in the experimental study of the transplantable tumors of the white rats and mice consists in the so-called immunity or resistance of certain animals to the growth of these implantations. This resistance apparently depends upon a number of various complex conditions, most of which as yet present no tangible explanation. It consists in every case of two components, one of which is the power of the implanted cancer cell to unlimited proliferation, and the other, the reactivity of the host. When a spontaneous tumor is inoculated, it grows successfully only in a small number of the animals used, frequently in less than 10 per cent. In subsequent inoculations with material taken from the implanted tumors, the success of the takes increases until it may reach fully 100 per cent., which indicates that the implanted cancer cell increases its proliferating power —its virulence. The fact, on the other hand, that during all these transplantations a certain number of animals retain their immunity to the growth of the tumor, clearly shows the influence of the reactivity of the host. This latter quality is considered by the majority of the investigators to be a natural characteristic of this animal, a natural immunity.

Immunity may also be induced artificially by various methods. Ehrlich (1), who first succeeded in rendering an animal resistant by artificial means, proceeded in the following manner. He inoculated a certain number of animals with a spontaneous hemorrhagic tumor, the cells of which were known to be of very low virulence and to absorb in nearly every animal without producing a growth. He

[1] Conducted at the expense of the George Crocker Special Research Fund. Read before the meeting of the American Association for Cancer Research, May 2, 1910. Received for publication, May 24, 1910.

then inoculated the same animals with a very virulent tumor with the result that a greater number of the treated animals were resistant to the implantation of the virulent strain than the control animals, and this resistance held good against the subsequent inoculation of nearly every other transplantable tumor, whether carcinoma or sarcoma. This phenomenon, which Ehrlich calls *pan-immunity,* appears to have a very wide application, and as is shown by the investigations of Bashford (2), Schoene (3), Borrel (4) and Bridré (5), may be induced by previous treatment, not only with tumor tissue, but also with different normal organ tissue of the same species of animals. Thus the immunity, or resistance to growth of tumor, does not appear to have a clearly specific character.

Ehrlich maintains further that animals on whom a successful inoculation of another tumor was done previously, *i. e.,* animals that had a tumor growth, appeared to be immune against a subsequent inoculation either of the same or another tumor. This observation was one of the main factors which served Ehrlich in the construction of his "atreptic" hypothesis of the immunity to tumor growth. Every implanted cancer cell, according to his idea, requires for the success of its further proliferation a specific food which it finds in the organisms of the host. When the first tumor is implanted into the animal, it takes up all the specific food available in the organism and consequently the cancer cell subsequently inoculated does not find in the host any of this specific food, and, therefore, no tumor growth takes place. This hypothesis of Ehrlich's will be considered with more detail later, but it must be stated here that both this theory and the phenomenon itself did not seem to find any corroboration among the subsequent investigators.

Hertwig and Poll (6), Gierke (7), and Borrel, on repeating these experiments, found opposite results. Borrel as well as Gierke explain this discrepancy by the difference in the technique employed in their investigations. They inoculated small pieces of the tumor under the skin, while Ehrlich used an emulsion. They believe that when an emulsion is used, only a small number of the cells proliferate, while the rest is absorbed and consequently produces an immunity against subsequent inoculation. When a small piece is inoculated, on the other hand, all the cells participate in the pro-

liferation, no artificial immunity is produced, and the subsequent inoculation is successful.

Nor does Borrel agree with Ehrlich's explanation of his results, that the immunity to the growth of cancer depends upon certain peculiarities in the distribution of food, but believes that it is analogous to the bacterial immunity and is caused by certain cytolytic anti-bodies circulating in the blood. He maintains that the first tumor grew in the animal because the anti-bodies did not have the time to develop, and when they do develop, they cannot interfere with the continued growth of the first tumor, but they may interfere successfully with the grafting of the second tumor. Ehrlich, on the other hand, explains the lack of corroboration of his finding by the fact that he always uses in his experiments a very virulent tumor for the first inoculation, a tumor which according to his ideas is best able to obtain all the specific food from the host, while the other investigators use tumors of less virulence, and there consequently remains in the organism a sufficient amount of specific food for the successful growth of the subsequent inoculation. This explanation of Ehrlich's seemed to find proof in the experiments of Apolant (8) with mixtures of emulsions of the two different tumors, sarcoma and carcinoma, for instance. At first he produced on such inoculation a tumor with the character both of carcinoma and sarcoma, but if one of the tumors used was more virulent than the other, then its character prevailed in the implanted tumor and the cells of the other tumor were suppressed. When all these facts are analyzed, the weight of evidence seems to tend to the possibility that the experiments of Ehrlich, Gierke, and Borrel may all be correct and the differences are not due to faulty methods, but to the fact that the conditions are different in these various experiments.

Two factors have to be accounted for in the consideration of the powers of resistance of the host to tumor growth. When a tumor is inoculated, the cells are grafted on the host, and whether the graft will be successful or not depends upon the ability of the host to supply a connective tissue stroma or "scaffolding," as Bashford calls it. It also depends upon the amount of the vascularization of this stroma. Russell (9) has shown that the difference in the morphological appearance between a successful and unsuccessful

tumor graft consists just in such a difference of the formation of a stroma. Indeed the investigations of the writer (10) seem to make it plausible that the fact of the success of the implantation of the cancers of the white rat and mouse, as compared with other laboratory animals, may be explained by such a great cellular reactivity of these animals. It seems feasible that during this stage of grafting or cohesion of the implanted cells to the host the result will be identical whether this cell possesses great power for proliferation, or simply a normal embryonic cell, as in the experiments of Askanazy (11). On the other hand, after the implanted cancer cell becomes ingrafted and is consequently able to develop its proliferating power, it again depends upon an interaction of a different nature between the implanted cell and the resistance of the host, whether there will take place unlimited proliferation and growth of cancer, or whether the cancer cell will succeed only in proliferating for a while, will form a small nodule and then become absorbed, and consequently will be just as innocuous to the organism of the host as an implantation of normal tissue. Every worker in cancer inoculation knows by experience that when pieces of a tumor are implanted in a set of animals, there will take place a tumor growth in a certain number of animals, in others the graft will disappear completely, while in others again there will appear a small nodule, which will later disappear. The two latter sets of animals are both ultimately immune to the inoculation of the tumor.

It stands to reason to suppose that resistance to the grafting of the implanted cell and resistance to its subsequent unlimited growth may be produced in a different manner. Such a difference is very well illustrated in the fact that, as was shown by Haaland (12), a tumor inoculated succeeds but poorly on a gravid animal, while, on the other hand, Herzog (13) has shown that when a tumor animal becomes pregnant, the tumor appears to grow faster. In other words, pregnancy produces a resistance against the grafting of tumor cells, while it enhances the proliferation of the tumor cells present in the organism. It is possible then that a tumor may find conditions favorable for its own growth and at the same time produce within the host an immunity to subsequent tumor implantation, as was the case in Ehrlich's experiment, while tumors used by the other investigators may not possess that property.

It is clear then that an artificial production of immunity against tumor growth depends upon a great variety of conditions, nor is there a unanimity of opinion as regards the *modus operandi* of this phenomenon. Borrel inclines to the view that the organism of an immune animal contains some anti-bodies of a peculiar nature, which restrain the growth, or rather exert a toxic influence upon the implanted cells, while Ehrlich and Bashford maintain that resistance to growth of implanted tumor is a purely cellular activity and that an artificial immunity can be induced only by inoculation of living cells.

But an analysis of the investigations on immunity does not seem to bear out fully the latter opinion. When an animal is immunized by a previous inoculation of normal tissue, all this tissue is completely absorbed by the time the tumor is inoculated, ten days later. Very instructive in this connection is the work of Woglom (14). He found that the spleen extirpated and then inoculated subcutaneously into the same animal induces resistance against growth of tumor. Extirpation of the spleen alone does not induce any resistance. If the resistance is caused in this case by the live functions of the cells of the spleen, they could act most effectively when the spleen was *in situ,* and the mice ought to have been naturally resistant. It is true that blood serum deprived of cells and tissue heated or crushed and frozen, do not appear to induce immunity, but this fact may be just as easily explained by the supposition that such a treatment of tissue is too severe and changes the chemical composition of those constituent parts of the injected cells, which induce the organism of the host to form anti-bodies. The weight of evidence seems to be against the idea that the cells used for immunization remain alive indefinitely in the new host and induce the resistance by their life functions. Ehrlich's atreptic theory resembles closely the exhaustion theory elaborated by Pasteur in explanation of bacterial immunity, but while it very ingeniously explains certain phenomena in the immunity to growth of tumor, it does not seem to be sufficient for the complete elucidation of all the data. It is also true that so far not a single experimental fact was adduced to prove the existence of anti-bodies in the blood serum of immune animals, nor was it possible to show the existence of

passive immunity (the experiments of Clowes and Baeslack (15) received no confirmation), but such negative results can hardly be of great value, since immunity to growth of cancer, while possibly similar, cannot by the nature of the process be identical with bacterial immunity.

In the study of the phenomenon of immunity to cancer growth, all the experience gained in the study of bacterial immunity should be closely followed, but new methods will have to be devised before the actual explanation will be reached. The fact that an animal organism creates anti-bodies against enzymes or any proteid matter introduced into the circulation is a certain indication of the fact that the existence of anti-bodies in the immune animal cannot be denied *a priori.* The aim of the present investigation consisted in the search for a substance which would not contain living cells and would still be able to immunize an animal against the growth of an implanted tumor. The work was done with Ehrlich's sarcoma of a white rat, for a transplant of which the writer is indebted to Dr. Simon Flexner. This tumor is of a very malignant type, takes in from 100 per cent. to 80 per cent. and grows to a very large size, frequently measuring two inches by one inch in about three weeks after inoculation.

A description of the different methods of treatment of animals employed in order to create a condition of immunity to cancer growth without the assistance of living cells will now be given.

Treatment with Arsazetin.—In view of the great inhibitory influence of the modern arsenical preparations (atoxyl and arsazetin) on trypanosomes and other animal parasites, and in view further of the frequent reports of the influence of arsenicals on human cancer, it is interesting to learn whether these drugs have any influence upon the growth of a transplantable sarcoma of a rat.

Sticker (16) reported that a combination of blood-serum and atoxyl retards growth of his transplantable sarcoma of a dog and occasionally even arrests completely its development. On the other hand, Uhlenhuth and Weidanz (17) state that atoxyl not only does not retard growth of carcinoma of a mouse, but even seems to enhance it.

Since the growth of sarcoma is more readily influenced than

carcinoma—and this fact may have explained the discrepancy between the results of Sticker and Uhlenhuth and Weidanz, the experiments were repeated on the sarcoma of the rat. Arsazetin was used instead of atoxyl, since it was found by Ehrlich (18) to be the more efficient of the two.

In our experiments, forty rats were treated for six weeks by hypodermic injections of one cubic centimeter of 4 per cent. solution of arsazetin, first every two days and then every four days. Three weeks after the beginning of the treatment the tumor was inoculated.

TABLE I.

Arsazetin.

	Treated animals.	Controls.
Number of rats inoculated with tumor	40	20
Number of rats surviving at final examination	32	20
Number of rats with tumors	28	19
Percentage of takes	87	95

As Table I shows, the arsazetin had no influence upon the growth of the tumor, though the quantity of the drug used was sufficient to impair considerably the general health of the animals. In nearly every animal a condition was created similar to the state of a waltzing mouse.

Treatment with Sodium Oleate.—It was stated above, that while no protective bodies were found in the blood of animals immune to the growth of cancer by the ordinary methods used in bacteriology, the possibility of the existence in the blood serum of some peculiar kind of anti-bodies cannot be excluded *a priori.* The investigations of Kyes (19) and Noguchi (20) have shown that a certain kind of cytolytic immune bodies become activated on the addition of lecithin and other lipoids, and Noguchi has further proven that sodium oleate seems to possess the strongest cytolytic action of all the lipoid substances. For this reason a certain number of animals were treated with an injection of a solution of sodium oleate previous to the inoculation of the tumor.

While the difference between the treated animals and the controls is not sufficient to indicate any actual influence of the lipoid used, the result is suggestive, and in view of the theoretical importance

of such a method of investigation, this part of the research is still being continued with different variations.

TABLE II.

Sodium Oleate.

	Treated animals.	Controls.
Number of rats inoculated with tumor	20	10
Number of rats surviving at final examination	18	10
Number of rats with tumors	14	10
Percentage of takes	77	100

Treatment with Autolyzed Tissue.—The investigations of Salkowski (21), M. Jacoby (22), P. A. Levene (23) and others, have shown that a great many of the so-called vital functions of the cell are due to the activities of the endocellular enzymes. These enzymes, while constituting an integral component part of the cell, may remain under certain conditions uninjured after the death of the cell. Furthermore, the investigations of Petry (24), Blumenthal and Wolf (25), C. Neuberg (26), Yoshimoto (27) and others have shown that the endocellular enzymes of the cancer cells seem to act differently both qualitatively and quantitatively from the enzymes of normal cells. In view of the further fact that the investigations of Müller (28), Opie (29), Blum (30), Conradi (31) and others have shown that these endocellular ferments play an important rôle in a number of pathological processes, and may be the means to which the organism resorts in order to elaborate protective substances—Conradi and Blum have shown that autolyzed tissue may act as an antitoxin—it seems feasible *a priori* that the resistance induced by normal mouse or rat tissue inoculated subcutaneously may also be due not to the function of a live cell, but to some peculiar type of an endocellular ferment. This assumption seems the more plausible since the best method of liberating these endocellular enzymes consists in the autolysis of tissues, and the tissue introduced under the skin in order to induce immunity is put under conditions most favorable for subsequent autolysis.

In view of all these considerations, a series of animals were treated with autolyzed tissue. The organ used for immunization

was the liver of "Nullers," that is, rats naturally resistant to tumor implantation. The tissue was kept under asceptic precautions at body temperature for two weeks, then the autolyzed tissue was mixed with about double the quantity of normal salt solution, ground thoroughly with sand, filtered, and one cubic centimeter of the solution injected subcutaneously.

TABLE III.

Treatment with Autolyzed Tissue.

							Total.	Control.
No. of rats inoculated with tumor.	10	10	10	10	10	10	60	60
No. of rats surviving at the final examination, 25 days after inoculation.	5	10	10	10	6	10	51	57
No. of days *before* inoculation that autolyzed liver was injected.	10	5	3					
No. of days *after* inoculation that autolyzed liver was injected.					9	9	12	
No. of rats without growth or with small abortive nodules.	2	7	5	7	4	9	34	6
No. of rats with tumors.	3	3	5	3	2	1	17	51
Per cent. of takes.	60	30	50	30	33	10	34	85

The results compare quite favorably with those of the investigators who induced immunity with normal tissue. Thus it seems possible to produce in rats a certain amount of resistance to growth of tumor by treatment with tissue of which the cells are killed, but the endocellular ferments apparently remain active. It is interesting to note here that autolyzed liver tissue seems to immunize equally well, whether used *before* or *after* the inoculation of the tumor, which fact may be of great importance in view of the possibility expressed above, that the resisting influence of the host may be of two kinds, one to the tumor implantation, the other to tumor growth.

Ligation of the Blood Vessels of the Spleen.—The spleen tissue gives the best results in immunization, as is shown by the work of Bridré, and it is also the most active tissue on autolysis, but the spleen of a rat is so small, that a sufficient amount of material for autolysis could not be collected. Another method was, therefore, tried. An abdominal incision was made on a rat and all the

vessels of the spleen were ligated and from four to twelve days after the operation the tumor was inoculated. In another experiment the operation was done nine days after the inoculation.

TABLE IV.

Ligation of Vessels of the Spleen.

	After the operation.	Before the operation.	Controls.
Number of rats inoculated with tumor.....	16	10	20
Number of rats surviving.....	14	10	20
Number of rats with tumors.....	9	10	19
Percentage of takes.....	61	100	95

The table shows that the immunizing influence of such a ligated spleen is not as strong as the influence of autolyzed liver injected subcutaneously, and also that this immunizing influence is exerted only on the grafting of the tumor and not on its continued growth. The action of the ligated spleen is most probably comparatively weak on account of the rapid absorption of the organ, which prevents the autolysis, and indeed no traces of such a spleen were found in the abdominal cavity two days after the operation.

Treatment with Alien Tissue.—The most important facts adduced by Ehrlich in support of his atreptic theory of tumor growth and immunity consist in his so-called zig-zag transplantations. He showed, namely, that when a tumor of a mouse is inoculated into a rat, it grows normally for eight or ten days and then ceases its growth and becomes absorbed, but if before the absorption takes place, the tumor is inoculated back into a mouse, it grows there to a large size. Ehrlich's explanation of this phenomenon is that a mouse tumor needs for its continued growth a certain food stuff X, which it can only find in the mouse organism. When it is introduced into the rat, it carries along from the mouse the X food, and as long as this storage of specific food lasts, the tumor grows; when it is exhausted, the tumor cells die, but when they are returned to the mouse, they find again the necessary food and proliferate. In order to give this contention a further test, a series of experiments was undertaken which consisted in the subcutaneous inoculation into a rat, of the normal skin and spleen tissue of a mouse, followed in a few days by a subcutaneous inoculation of

the tumor. The aim of this treatment was to accustom the tumor cells to mouse tissue and then to observe whether such a rat tumor, which had the opportunity to obtain during its growth the food supplied by the inoculated normal mouse tissue, would not grow more readily when subsequently inoculated into a mouse.

The results of this investigation were negative, but the extremely interesting fact was observed that a certain number of the rats treated with mouse tissue appeared immune against growth of the rat sarcoma. The following table will illustrate this phenomenon.

TABLE V.

Treatment with Alien Tissue.

	Treated animals.	Controls.
Number of rats inoculated with tumor	40	40
Number of rats surviving at final examination	27	40
Number of rats with tumors	10	36
Percentage of takes	37	90

Similar positive results were obtained recently by C. Lewin (32), who succeeded in immunizing rats with mouse tumors and *vice versa*. This possibility to immunize an animal against growth of tumor by treatment with alien tissue seems to indicate that Ehrlich's atreptic theory, while possibly of value in the explanation of certain facts, does not seem to have a universal application.

Organ cells of a mouse do not possess, nor do they require any specific food of the rat, which is the reason why a rat tumor fails to grow indefinitely in a mouse. When a rat is immunized against growth of a rat tumor by previous treatment with mouse tissue, such a failure to grow cannot be ascribed to the lack of proper nourishment within the host, since the previously inoculated mouse cells could not have absorbed such food.

Another important conclusion which may be reached from this series of experiments consists in the fact that this immunization cannot be due to life activities of the cells, since alien tissue could not remain alive for thirteen days after it was introduced under the skin. Furthermore, this phenomenon is of practical value in the further pursuance of this investigation. It is extremely difficult, as was stated above, to obtain the necessary amount of tissue

from these small animals for autolysis. Since it was found that alien tissue may be used for immunization, organs of large animals (dogs) are being utilized for autolysis, and on addition of tumor tissue of a rat, heterolytic action is obtained. This is of great importance in a number of pathological processes and also in cancer, as is shown by the investigations of C. Neuberg and others. But these experiments are not yet sufficiently far advanced for conclusions to be drawn. The same is true for the experiments undertaken with the aim in view of obtaining in large animals autolytic antiferments and possibly, therefore, of inducing a passive immunity to the growth of the transplantable tumors.

The results obtained in this investigation are not final; a great deal more work must be done on different tumors. The difficulties encountered in such an investigation is well illustrated by a very recent statement of Abderhalden and Pringsheim (33), that though using the best methods for liberating endocellular enzymes, they occasionally do not find any enzymes in the solution, but the latter apparently become adherent to the filtrate and sand. Consequently, while positive results in our research are convincing, negative results may always be due to insufficient technique. Different means then must be employed to liberate the greatest amount of ferments and entirely new methods will probably be created before conclusive results will be reached, but the viewpoint appears to be correct and the work seems to be pursued in the right direction. Expressions like "life activity of the cell" or "cellular activity" hardly have any significance in biology; the active constituent part of the cell must be found, and autolysis may be the road to it. The idea, at least, is capable of stimulating further research.

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THE RELATION OF FATTY DEGENERATION TO THE OXIDATION OF PURINES BY LIVER CELLS.[1]

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In studies of liver function and of various problems of metabolism, attempts have frequently been made to reduce or eliminate the action of the liver by means of various poisons, with the hope of ascertaining its influence upon nitrogenous and carbohydrate metabolism. Phosphorus has chiefly been used for this purpose, although other steatogenic poisons have also been tried, and, less often, specific hepatatoxic and hemotoxic sera. Also, the changes in metabolism noted in natural disease processes, notably acute yellow atrophy, chloroform necrosis of the liver, and puerperal eclampsia, have been ascribed to the impaired hepatic function assumed to result from the extensive structural changes that are present in the liver in these conditions. While the earlier investigators for the most part considered that marked disturbances in nitrogenous metabolism resulted from the hepatic lesions, the later observations, particularly those of Jackson and Pearce[2] and Underhill and Kleiner[3] fail to substantiate this view.

Jackson and Pearce found that with focal necroses of the dog's liver due to hemotoxic sera there is but a slight change in the ammonia excretion, but a marked rise in the undetermined nitrogen; in "diffuse degeneration" there is more relative increase in the ammonia nitrogen, and little rise in the undetermined nitrogen. "In necrosis, of even considerable extent, the total nitrogen is greatly augmented, as is also the rest-nitrogen; while the production of urea, on account of the persistence of normally functionating liver cells, remains relatively unchanged. This factor of safety

[1] Read before the Association of Pathologists and Bacteriologists, Washington, May 3, 1910. Received for publication May 26, 1910.

[2] Jackson and Pearce, *Jour. of Exper. Med.*, 1907, ix, 521–587.

[3] Underhill and Kleiner, *Jour. of Biol. Chem.*, 1908, iv, 165.

possessed by the liver is, we think, one of the most important results brought out by this investigation and must be given great weight in any consideration of the chemistry of hepatic disturbances."

They also found that uric acid elimination is increased after the hemotoxic serum is injected into dogs, and this increase is ascribed to the destruction of the nucleoproteins of the necrotic cells. Allantoin elimination is also increased. These last two observations indicate that the power of the injured liver to hydrolyze nucleoproteins and to oxidize the free purines into uric acid and allantoin is not destroyed, and apparently it is not seriously impaired. They conclude that their results "do not indicate that the injection of a hemotoxic serum causes a prolonged or a decided decrease in the oxidative power of the hepatic cell. As a whole, the organ continues to perform its functions in the normal way."

Underhill and Kleiner poisoned dogs with hydrazine, which is characterized by causing profound fatty changes in the liver, and studied the subsequent changes in metabolism. They found that the partition of urinary nitrogen and sulphur is only slightly different from that which obtains during inanition, and as dogs poisoned with hydrazine refuse all food, they believe that such urinary changes as are observed are due to this starvation rather than to the hepatic changes. One striking feature of both inanition and hydrazine poisoning is the elimination of much allantoin in the urine. Since this paper was written, it has been shown by several observers[4] that in the dog allantoin is produced chiefly, if not entirely, in the liver by the action of the oxidizing enzyme, *uricase*, upon uric acid. Therefore, the experiments of Underhill and Kleiner indicate that even in the extreme fatty degeneration which occurs in the liver in hydrazine poisoning, the liver is able to oxidize endogenous purines into allantoin. They agree with Jackson and Pearce in emphasizing the efficiency of the "factor of safety" of the liver in preventing noticeable deficiency in its function as a result of extensive pathological changes.

Through the kindness of Professor Underhill, I was given the opportunity of studying the tissues of his dogs which had died from

[4] For literature, see *Jour. of Biol. Chem.*, 1910, vii, 171.

hydrazine poisoning, and to make a further study of the histological changes produced in the tissues of mammals by this poison.[5] It was found that hydrazine is a peculiarly specific poison for the cytoplasm of hepatic cells, causing extreme fatty degeneration of these cells with almost no nuclear changes and without causing the death of the cell, while all other organs are practically unaffected. The changes begin in the center of the lobule, and when the intoxication is not too rapidly fatal, all the cells but a narrow peripheral margin are crowded with fat droplets to the utmost degree; these outer cells also always show more or less fat, but in no case did all the cells show the extreme changes observed in the central cells. It is to be presumed that it is these cells that constitute the "factor of safety" in hydrazine poisoning, although the fact that the nuclei of even the most fatty cells are left practically intact may also be of some importance.

It is well recognized that fatty metamorphosis of cells occurs when their supply of oxygen is reduced from either local or general causes, and hence it has been suggested that poisons which cause similar fatty changes may act by interfering with the oxidative processes of the cell,[6] presumably by inhibiting or destroying the oxidizing enzymes, although other methods of causing decreased oxidation are conceivable. Certain observations recently made upon the processes of purine destruction suggested that an examination of the influence of steatogenic poisons upon the purine enzymes might give interesting results, both from the standpoint of the problems of purine metabolism and of fatty degeneration. It will be recalled that the known steps of purine metabolism involve first a series of deamidizations, and then of oxidations. By the oxidation processes, hypoxanthine and xanthine are oxidized into uric acid, and in turn the uric acid is oxidized into allantoin and carbon dioxide. This last step is performed by a specific enzyme, uricase, which is of strictly limited occurrence, being found only in mammals, and in them only in the liver or kidney, generally the former alone. Man, indeed, is entirely devoid of this enzyme so far as

[5] The Pathological Anatomy of Hydrazine Poisoning, *Jour. of Exper. Med.*, 1908, x, 457.

[6] Wells, Chemical Pathology, Philadelphia, 1907, p. 341.

can be demonstrated by experiments *in vitro*.[4] Not only is this enzyme of remarkedly limited distribution, but, in harmony with this fact, it is the last of the enzymes of purine metabolism which appears in the development of the mammalian organism; in the pig it apparently does not appear until after birth.[7] Evidently, then, uricase is a highly special enzyme, of late development in both the evolution of the animal kingdom and of the individual mammal. On this basis, we should expect it to be, in harmony with the general rule of pathology, one of the first to suffer under the influence of injurious agencies or conditions. This, together with the fact that we have reasonably accurate quantitative chemical methods for the estimation of the uricolytic power of tissues, made the study of the influence of steatogenic poisons upon this particular oxidizing enzyme seem of especial interest.

In order to render the fatty changes as extensive as possible, advantage was taken of the fact that in hydrazine we have a poison which causes a profound change in the center of the liver lobule, while phosphorus attacks first and more extensively the periphery of the lobule. As hydrazine affects only the liver, it is possible to secure a high degree of fatty degeneration before death occurs from this poison, and by giving the more lethal phosphorus only after extensive central changes have been produced by the hydrazine, a remarkable degree of fatty change can be produced, with practically every cell in the liver lobule showing the most extensive involvement.

Livers rendered fatty in this way were removed from the animals immediately after death, and were studied as to their ability to destroy uric acid *in vitro;* they were also examined microscopically both with specific fat stains and with hematoxylin and eosin. The methods and results are described in the following protocols.

Experiment I.—Dog, young, weight 10 kilos. Given on the first day 0.15 grm. hydrazine sulphate subcutaneously. After forty-eight hours no symptoms had been shown, and 0.3 grm. more was given. Twenty-four hours later the dog was a little sick, and 1 c.c. of a strong solution of phosphorus in oil was given. The next day the dog was still only slightly sick, and 2 c.c. more of the phosphorized oil was given. Death occurred five days after the first dose of hydrazine, and forty-eight hours after the first dose of phosphorus. Autopsy showed the liver to be quite fatty and friable.

[7] Mendel and Mitchell, *American Jour. of Physiol.*, 1907, xx, 97.

Microscopical examination shows a high degree of fatty change (Sudan III) which involves the central parts of the lobule to a greater degree than the periphery, but there are no cells which do not contain more or less fat in fine droplets, and even the epithelium of the bile ducts is full of fat. Each liver cell, except a few at the very periphery, is packed with fat in droplets generally from a fourth to a half the diameter of the nucleus, which is entirely obscured when no nuclear stain is used. Even at the periphery of the lobules every cell contains many fine droplets of fat, but they are not so packed as in the cells at the center. Sections stained with hematoxylin and eosin show a greater difference between center and periphery, and it is noticeable that even in the most fatty cells the nuclei usually stain well, and there is no evidence of necrosis, thrombosis, or leucocytic infiltration. Kupffer cells and capillary endothelium seem unaffected, except for a few vacuoles. In the center of some lobules the blood spaces are dilated, as if replacing liver cells which are here reduced to a mere cobweb.

One hundred and twenty grm. of liver tissue were ground to a pulp, first in a hashing machine and then in a mortar, until it could all be passed through cheese cloth, except a few shreds of fibrous tissue and vessels. To this emulsion was added 0.308 grm. uric acid, which had been dissolved in boiling water, by adding the minimum amount of sodium hydrate necessary, and cooled. The whole material was made up to 600 c.c. with toluene water. For control 110 grm. of liver from a freshly killed normal dog of approximately the same size and weight were prepared in exactly the same manner, and the same amount of uric acid was added.

It was considered advisable, in order to make the results of the comparison more valuable, to use somewhat more tissue from the poisoned than from the normal liver, partially to compensate for the increase of weight due to fat. As analyses of similar fatty livers have shown that the fat-free proteins constitute but 10 to 11 per cent. of the weight of the fresh liver, as compared with 17 to 21 per cent. in normal livers, it is evident that in these experiments the amount of active tissue used was somewhat less with the poisoned than with the normal liver.

Autolysis at 35° C. was begun, with an active current of air drawn through the emulsion after it had been saturated with toluene and moisture by bubbling through a bottle of toluene and water kept at the same temperature. Samples of 100 c.c. each were withdrawn at the end of four, six, nine, twenty and twenty-nine hours. Each sample was diluted to 300 c.c., and the protein coagulated by boiling and adding a few drops of dilute acetic acid. The filtrate from the coagulum was heated to boiling and the purines precipitated by the Kruger-Solomon method. In only the first, or four-hour sample, was a definite although very minute precipitate obtained; with the other samples a doubtful turbidity at the most was observed. After decomposing these slight precipitates with hydrogen sulphide, and concentrating the filtrate, but a few crystals of uric acid could be obtained from either of the first two (four-hour) samples, not sufficient for weighing; these gave a distinct murexide test. No uric acid was present in any of the other samples.

Therefore, 100 grm. of dogs liver had destroyed almost totally 0.3 grm. of uric acid in 4 hours, under the conditions of the experiment, there being no appreciable difference in the results with normal or fatty liver tissue.

EXPERIMENT II.—A young dog, weighing about 9 kilos, received an injection of 0.18 grm. hydrazine sulphate. Two days later, there being no marked symptoms, 0.24 grm. hydrazine sulphate and 1 c.c. phosphorized oil were injected. Twenty-four hours later it was quite sick, and 2 c.c. more of the phosphorized oil was given. It died four days after the first injection, and the liver was found to be extremely fatty.

Microscopical Examination.—Sections stained with Sudan III show a more extensive and complete fatty change than was present in Experiment I. Every cell, alike in all parts of the lobule, is packed with fat droplets, including even the bile duct epithelium; there is no appreciable difference between the cells of periphery and center of the lobule as regards the fat content, but in some lobules there seems to have been a partial replacement of the central cells by blood. Hematoxylin and eosin staining shows that the peripheral cells have retained more cytoplasm than the central cells, although even the best show many fat spaces. In the centers of the lobules there remains little cytoplasm on many of the cells, and not rarely nuclear fragmentation is observed; dilated sinusoids take the place left by the reduced liver cells. A few liver cell nuclei in mitosis are found.

In order to postpone the total destruction of the uric acid and thus lengthen the period of observation, but 60 grm. liver were used, with 0.524 grm. uric acid, nearly twice as much as was used in the first experiment. For control 50 grm. of normal liver with the same quantity of uric acid was taken. The experiment was conducted as before, 100 c.c. samples being drawn off at the intervals stated. The results obtained were as follows:

Duration of autolysis.	Uric acid recovered from 100 c.c. sample of normal liver.	Poisoned Liver.
Initial	0.087	0.087
1 hour	0.038	0.036
3 hours	0.033	0.032
6 hours	0.002	trace
12 hours	0	0
24 hours	0	0

EXPERIMENT III.—Adult terrier, weight 12 kilos. Given 0.18 grm. hydrazine sulphate, and after forty-eight hours, as the dog did not seem sick, another dose of the same size was injected. Twenty-four hours later 1 c.c. of phosphorized oil was given, which caused the animal to be quite sick after twenty-four hours when the same dose was repeated. Death occurred five days after the first injection, and the liver was found to be extremely fatty.

Microscopical Examination.—The description of the liver in Experiment II will apply to the findings in this case, the only difference being that the amount of fat is, if anything, a trifle less, but the difference is insignificant.

In this experiment 55 grm. samples of the fatty liver and 50 grm. samples of normal liver were used, and two samples of each were taken. To one pair (A) was added 0.567 grm. uric acid to both the normal and the poisoned liver, and to each of the other pair (B), 0.498 grm. of xanthine. Autolysis was con-

ducted at a lower temperature than in the other experiments, about 32° C. One hundred c.c. samples were removed at intervals from each of the four flasks, and analyzed as before, with the following results.

Duration.	(A) Uric acid recovered from each 100 c.c. emulsion of		(B) Xanthine recovered from each 100 c.c. emulsion of	
	normal liver.	fatty liver.	normal liver.	fatty liver.
Initial	0.094	0.094	0.083	0.083
2 hours	0.067	0.065	0.0635	0.065
4½ hours	0.042	0.057	0.045	0.056
8 hours	0.005	0.015	0.042	0.045
20 hours	0.0	0.0	0.010	0.015
44 hours	0.0	0.0	trace	trace

The xanthine samples contained practically no uric acid, the merest trace being obtainable by Horbaczewski's method. Evidently, then, the uric acid formed from the xanthine is destroyed as fast as formed.

EXPERIMENT IV.—An adult maltese cat, weight 3900 grm., was given 0.15 grm. hydrazine sulphate, which caused it to become quite sick after two days, and on the third day it was given 0.15 grm. hydrazine sulphate and 1 c.c. phosphorized oil. It died four days after the first injection, and the liver was found to be extremely fatty, soft and almost pulpy.

Microscopical Examination.—The amount of fat present in the liver of this cat exceeded that observed in any of the dogs, especially in that the peripheral cells were quite as fatty as the central cells. With hematoxylin and eosin the vacuolization of the peripheral liver cells was also found to be about equal to that in the center of the lobule, but in the center of the lobule many of the cells contain no nuclei, the nuclei remaining being chiefly those of the Kupfer and endothelial cells.

A normal cat of similar age and weight was killed, and 50 c.c. of its liver was used as control for 55 c.c. of the fatty liver, adding to each 0.47 grm. uric acid and making up to 600 c.c. In this experiment the autolysis was conducted at 40° C. The result of the autolysis of the samples was as follows:

Duration.	Uric acid recovered.	
	Normal liver.	Fatty liver.
Initial	0.079	0.079
2½ hours	trace	trace
4 hours	trace	trace
6 hours	0	0

The amount obtained in each of the two and one half hour samples was too small to weigh satisfactorily, not being over 5 mg., while in the four hours experiment but the smallest trace, barely enough for the murexide test, was obtained.

Therefore under the conditions of this experiment 50 grm. of cats' liver, representing about 10 grm. of solid substance, and probably not more than 5 grm. of dry, fat free liver tissue, has destroyed almost totally $\frac{1}{20}$ of its dry weight of uric acid in two and a half hours.

The remaining liver extract was placed in dark bottles, covered with a heavy layer of toluene, tightly stoppered, and placed in the incubator at 40° C. to see if resynthesis would occur, as stated by Ascoli and Izar, and, if so, whether there was any difference between the normal and the fatty liver. After three days a sample was drawn off from each bottle, the remainder being placed in smaller bottles of suitable size to eliminate air space, and left in the incubator. No uric acid could be recovered from either of the samples removed at the end of three days. After fourteen days the remainder of the material was analyzed, and again no uric acid could be obtained from either sample.

EXPERIMENT V.—A healthy rabbit, weight about 3000 grm., was given 0.06 grm. hydrazine sulphate on the first and third days, and the same plus 1 c.c. phosphorized oil on the fourth and fifth days. It did not become sick until after three days, and died on the fifth day. The liver was extremely fatty.

Microscopical Examination.—The same uniform high degree of fatty change is present as with the cat, differing only in that there is less loss of liver cell nuclei in the central portions of the lobules.

Of this liver 45 grm. was used in contrast with 40 grm. of normal liver from a healthy rabbit of the same size and age. To each was added 0.49 grm. uric acid and toluene water to make 600 c.c. and the autolysis was conducted at 40° C., with the following results:

	Uric acid recovered.	
Duration.	Normal liver.	Fatty liver.
Initial	0.082	0.082
2 hours	0.047	0.045
4 hours	trace	trace (more than in control)
8 hours	0	0

Here we have destruction of 0.5 grm. uric acid by 40 grm. of rabbit liver, or about 1 to 16 of solid substance, practically complete in four hours and equally as rapid with the highly fatty liver as with the normal one.

After continuing the autolysis for twenty-four hours with air, the remaining material was put into tightly stoppered bottles and left without air at 40° C. for thirty days, at the end of which time analysis revealed no appreciable quantity of uric acid in either sample.

SUMMARY.

These experiments show that it is possible to cause, by the proper use of hydrazine and phosphorus, a very high degree of fatty change in the liver with a minimum of necrosis, involving alike both peripheral and central portions of the lobule and thus eliminating the "factor of safety" or residual normal cells. Nevertheless, it is found that such fatty liver tissue has not lost in the least its power to oxidize uric acid when acting upon it *in vitro* with abundant air supply, nor is the power to oxidize xanthine to uric acid noticeably diminished. Since these two enzymes, and espe-

cially the uricolytic enzyme, are about the last to appear in the development of the animal kingdom and also of the individual mammal, it is to be expected that they will be among the first of the enzymes to be destroyed by injurious agencies, but evidently they are not affected by conditions that lead to the highest degree of fatty metamorphosis of the cytoplasm of the hepatic cells. While these experiments merely prove that extreme fatty degeneration does not destroy or appreciably diminish the power of liver cells to oxidize uric acid and xanthine *in vitro*, yet they suggest that in general fatty degeneration is probably not essentially incompatible with a high degree of metabolic activity by the affected cells. It is possible that in the living tissue functional activity may be decreased secondarily by fatty metamorphosis, as, for example, by the enlarged fatty cells compressing the capillaries and reducing circulatory activity, or by modification of diffusion through the cell by the fat deposits, independent of any deleterious influence upon the cellular enzymes themselves. Other experiments will be required to determine whether steatogenic poisons leave intrahepatic enzymes other than the uricase equally unaffected, but as it has already been shown by Jacoby[8] that the autolytic enzymes and aldehydase in the liver cells are not destroyed by phosphorus poisoning, and Abderhalden and Schittenhelm[9] found that peptids are hydrolyzed fully as rapidly by phosphorus livers as by normal livers, it seems probable that extreme fatty metamorphosis is not associated with any serious injury to the enzymes by which the liver cells perform their metabolic functions, with the possible exception of those enzymes concerned with the metabolism of fats.

[8] Jacoby, *Zeit. f. physiol. Chem.*, 1900, xxx, 174.

[9] Abderhalden and Schittenhelm, *Zeit. f. physiol. Chem.*, 1906, xlix, 41.

EXPERIMENTAL YAWS IN THE MONKEY AND RABBIT.[1]

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PLATES LIII AND LIV.

INTRODUCTION.

Yaws or frambesia has always been of particular interest on account of its possible relation to syphilis, and since the discovery of its cause it has been studied with renewed activity. The discovery of *Treponema pertenui,* by Castellani (1) in 1905, was a direct sequel to Schaudinn's work on syphilis, and both Schaudinn and Castellani considered the organisms of the two diseases morphologically identical. More recently, minute differences in form have been pointed out by Prowazek (2) and Russell (3); but whether or not they are to be accepted as sufficient for differentiation, there is no doubt, on the basis of immunity experiments on monkeys (4) and man (5), that the two diseases are distinct. In Castellani's words, "Yaws is not syphilis any more than leprosy is tuberculosis."

In the recent study of syphilis, a great advance has been made by Parodi (6) and Uhlenhuth and Mulzer (7), who have demonstrated the possibility of producing a syphiloma in the testicle of the rabbit. As Uhlenhuth and Mulzer say, this lesion gives us a convenient and rich source of spirochetæ, in pure culture, with which cultivation and immunity experiments can be carried out.

Several months ago, I undertook some work on syphilis in the rabbit's testicle and, as a case of yaws was under my observation

[1] Read (by invitation) at the annual meeting of the American Society of Pathologists and Bacteriologists, Washington, D. C., May 5, 1910. Received for publication June 1, 1910.

at the time, I thought it worth while to attempt to infect rabbits with the spirochetæ of yaws as well as with those of syphilis. This attempt has been successful and I have been able to observe a parallel series of infections.

The spirochetæ of yaws came from a colored soldier returning from the Philippines (8). This soldier, forty years of age, had a well-marked case of yaws consisting of three separate general eruptions and marked joint symptoms. Spirochetæ in small numbers were found in the serum from the yaws on two occasions with the dark field microscope (Plate LIII, Fig. 1).

EXPERIMENTS.

On Dec. 17, 1909, a monkey (*Macacus rhesus* 1) was inoculated by rubbing an excised piece of a yaw into an abraded surface of the right eyebrow. The abrasion healed completely in a few days. On Jan. 7, 1910, 24 days after inoculation, a red and slightly scaly patch was noticed on the right eyebrow, and in a few days a well-marked red and edematous ridge of tissue developed. Spirochetæ in small numbers were repeatedly found in fluid expressed from this lesion. The growth invaded the upper eyelid, ulcerated, and on Feb. 2 showed a typical yellow crust formed by the rich serous discharge from fungoid granulations (Plate LIV, Fig. 2).

Three rabbits were inoculated in the testicle with serum from the lesion in the monkey. All became infected as follows: Jan. 27, the unbroken surface of the edge of the monkey's yaw was slightly abraded, a drop of clear serum was expressed, taken up in a capillary pipette and expelled by air pressure into the substance of the right testicle of a large white rabbit, A. Weekly examinations were made and no change was noticed until Feb. 24, 28 days after inoculation, when the right testicle was found uniformly increased in size so that it could not be retracted; it was firm throughout but more resistant at the upper part. On Feb. 25, the resistant area was punctured with a sterile capillary pipette and a drop of clear gray fluid obtained which, under the dark field microscope, showed a great number of long active spirochetæ of the *pertenuis* or *pallida* type. The second rabbit, A^1, inoculated in the same way, showed an enlarged testicle containing a small round nodule rich in spirochetæ after 43 days. The third rabbit, C, became infected after 52 days.

With material from the first rabbit, A, the disease was *reproduced in the monkey and continued in a second generation in rabbits.*

March 9, a drop of serum from the testicle of Rabbit A, rich in spirochetæ, was rubbed into the eyebrow of a monkey (*Macacus rhesus* 2). In 16 days a red papular area appeared and soon became markedly elevated, edematous and slightly scaly. Spirochetæ were readily found in the expressed fluid.

Feb. 25, 8 rabbits were inoculated with fluid from the testicle of Rabbit A; 3 with undiluted fluid and 5 with fluid diluted with 3 parts of salt solution. One of the 5 died after 14 days of rabbit septicemia, and there was no change

in the testicle and no spirochetæ were found. Two of the rabbits inoculated with undiluted fluid developed lesions rich in organisms 32 and 42 days after inoculation. One of the 4 remaining showed an infection after 52 days. All these rabbits were undersized and gray or brown.

For the third generation larger animals were selected. March 29, 2 large white rabbits were inoculated in the right testicle by syringe with about .5 c.c. of a mixture of about 3 parts of salt solution and one part of fluid from a nodule in the testicle of Rabbit 7 (second generation). April 18, after 20 days, one showed an enlarged testicle with a distinct nodule very rich in active spirochetæ. April 22, after 24 days, the other showed the same. March 31, Rabbit 7, of the second generation, was castrated under ether anesthesia; a white edematous tumor was found occupying the middle of the testicle. Small pieces of this tumor were introduced in the testicles of 4 large brown rabbits with a trochar and canula; on April 22, after 22 days, Rabbit 14 showed a definite nodule rich in spirochetæ. Two other animals, Nos. 15 and 17, showed lesions after 36 and 22 days.

Thus the spirochetæ of yaws have been transferred from man to the monkey and from the monkey through three generations in rabbits. Eight out of sixteen rabbits used in this series have become infected. The incubation period averaged forty-one days in the first generation, forty and a half days in the second generation and twenty-four and eight-tenths days in the third generation, thus showing a shortening of sixteen days in the third passage. The series is being continued and, by using care in the selection of animals, can probably be continued indefinitely. Large animals are more suitable than small and in case of both syphilis and yaws the order of susceptibility seems to be white, gray, brown and black. I do not know yet which is the best medium for transfer, undiluted serum, salt solution, citrate solution or the bits of tissue: all have been successful. Inoculation in the anterior chamber of the eye has as yet failed of results. In tapping the testicle, if the point of a moderately strong capillary pipette is guided into the nodule, spirochetæ, in pure culture, can be obtained in myriads; in the testicle away from the nodule they are found in small numbers. They are readily stained by Giemsa's method (Plate LIV, Fig. 3). The dark field microscope is almost indispensable in handling a large number of animals. Swelling of the testicle and edema of the tunica vaginalis may result from frequent tappings, but no mixed infections of the testicles have occurred. The general health of the rabbits does not seem to suffer.

The Lesion of Yaws in the Rabbit's Testicle.—Three rabbits were castrated, under ether, thirty-four, forty-eight and sixty days after inoculation and the tissue used for histological study. If the testicle is examined soon after a nodule is palpable, the nodule is found to be a whitish edematous tumor fairly well differentiated from the rest of the testicle which appears normal. On incision, the edges of the growth roll back and a good deal of serum escapes. The tissue appears homogeneous. Sections show a picture of an interstitial orchitis; the tubules are small, disintegrating or replaced by an edematous connective tissue containing an infiltration of small round and endothelial cells. New polymorphonuclear cells are seen. In sections stained by Levaditi's method spirochetæ are found in large numbers in and around the tubules and in the interstices of the connective tissue (Plate LIV, Fig. 4). In the testicles examined after forty-eight days the infiltrating cells had for the most part disappeared and after sixty days the connective tissue was more fully organized and no spirochetæ were found. Repeated tapping of these testicles may have somewhat changed the natural history of the lesions but apparently the lesion resolves in time, leaving an atrophied organ.

Serum Reactions.—The complement fixation reaction has been tried on the yaws rabbits and has been positive in several cases.

.1 c.c. of serum heated at 56° C. for 15 minutes, acetone insoluble liver extract as antigen, and the human corpuscle and rabbit serum hemolytic system were used.

White Rabbit No. 6, second generation. Feb. 25, inoculated with undiluted serum from Rabbit A. March 1, serum negative; April 8, nodule palpable (42 days), spirochetæ present; April 9, serum negative; April 19, serum positive (53 days).

In the second case a positive reaction led to a reëxamination and the finding of spirochetæ.

Gray Rabbit No. 3. Feb. 25, inoculated with diluted serum from Rabbit A. Serum negative; April 18, testicle increased in size; no definite nodule (52 days); April 19, spirochetæ not found in one slide with dark field microscope; April 20, serum taken on April 19, positive, April 21, spirochetæ found (55 days).

The patient's blood was negative on four examinations, November 20, December 14, February 23 and March 15, after the second and third eruptions and after treatment with potassium iodide. Both monkeys have failed as yet to give the reaction. There are

two examples of the reaction in yaws on record, in both of which it was positive. Bruck (9) gives the case of a thirty-year-old Malayan who had yaws five years before. Hoffman (10) records a case of a native of Suaheliland with a positive reaction at the time of the eruption. The diagnosis was doubted by Ziemann. If, as seems probable, this reaction is not a specific immunity reaction, but rather a result of the breaking down of certain kinds of tissue, a good deal may possibly be learned from the study of the reaction in closed lesions in such a specialized organ as the testicle.

Syphilis in the Rabbit's Testicle.—A comparison of yaws with syphilis in the rabbit brings out several points of importance. January 27, a patient with mucous patches of the mouth and anus, untreated for three months, and with a clear history of a primary sore and a secondary eruption, was secured through the kindness of Dr. Seay. A patch on the lower lip was cleaned, aspirated with a suction apparatus and a few drops of serum containing numerous spirochetæ was injected with a capillary pipette into the left testicle of five large rabbits.

Feb. 28, testicle of Rabbit E enlarged; cannot be retracted; nodule felt; spirochetæ numerous (32 days).

Two other rabbits showed infection after 35 and 56 days.

Jan. 31, 5 rabbits inoculated from mucous patch; 2 were infected after 45 and 64 days. One of these rabbits is especially interesting as the infection travelled over to the uninoculated testicle and the Wassermann reaction appeared, disappeared and reappeared.

Jan. 31, large gray rabbit, G, inoculated right testicle from mucous patch; March 17, nodule felt in epididymis (46 days); March 18, spirochetæ present; March 19, castrated, circumscribed syphiloma size of an olive involving epididymis and testicle. March 29, serum positive, .50 and .15 c.c.; March 30, left testicle slightly enlarged, punctured, spirochetæ not found. April 4, serum negative. 1 c.c.; April 8, left testicle punctured, spirochetæ not found; April 9, serum negative, .1 c.c.; April 11, left testicle punctured, spirochetæ not found; April 15, left testicle: small nodule, spirochetæ not found; serum positive, .1 c.c., 29 days after appearance of lesion in *inoculated* testicle. April 22, left testicle; definite nodule in epididymis, *spirochetæ plentiful.*

The route of infection in this case was probably by the lymphatics, but may have been by the blood, as Truffi (11) has recorded an infection of the anterior chamber of the eye following an inoculation of the scrotum. One of the yaws rabbits has also shown this migration of spirochetæ to the opposite testicle; the lesion developed thirty days after castration of the inoculated testicle.

Treponema pallidum has also been carried through a second and third generation in rabbits.

March 9, grey rabbit, Q, inoculated left testicle, with serum from testicle of Rabbit E (first generation); April 14, nodule in testicle (36 days); April 15, spirochetæ present, April 15, serum positive, .1 c.c.; March 8, white rabbit, L, inoculated with serum from testicle of Rabbit G (first generation) in 2 per cent. citrate solution; April 15, testicle enlarged, nodule, spirochetæ present (31 days); serum negative.

One other rabbit of this generation became infected after forty-three days; three rabbits failed to take the infection.

Three rabbits of the third generation have become infected with an average incubation period of twenty-four days. The fourth generation is under observation. The average incubation period for the first generation was forty-four days, and for the second generation thirty and seven-tenths days.

Treponema pallidum transferred from the Rabbit to the Monkey.—March 9, monkey (*Macacus rhesus* 3): left eyebrow abraded and serum from testicle of Rabbit E rubbed in; spirochetæ present, first generation in rabbit from mucous patch.

April 5, slightly red area on left eyebrow; April 6, lesion very scaly, spirochetæ present (28 days); April 11, flat red area covered with fine scales; April 21, serum negative.

COMPARISON OF THE LESIONS.

The difference in the lesion of syphilis and yaws in the monkey seems to be the surest way to differentiate the organisms. In yaws the incubation period is two to three weeks, the lesion is elevated, slightly scaly, and very edematous; in syphilis the incubation period is about four weeks, the lesion is flat, dry and very scaly. According to Prowazek and Russell, *Treponema pertenui* is slightly thicker than *Treponema pallidum,* less rigid, less regular in its twists. With an abundance of material at hand, comparative observations have been made with the dark field microscope. *Treponema pallidum* seems quite constantly to show more active corkscrew motion; the twists seem more regular and not so sharp and deep; but occasionally an unknown specimen has been classified wrongly on these points and no other differences seem more reliable. The lesion in the rabbit seems to differ only in degree; the nodules in syphilis seem larger and have a necrotic center; histologically, there is evidence of a more active inflammation.

SUMMARY.

1. Rabbits can be infected in the testicle with the spirochetæ of yaws as well as with those of syphilis and the infection can be continued through successive generations in pure culture.

2. The infection shows itself by enlargement of the testicle and the presence of a nodule varying in size from that of a pea to that of an olive. The infection consists in a necrosis of the tubules, an infiltration of round cells and the new formation of an edematous connective tissue.

3. The complement fixation reaction occurs in rabbits infected with the spirochetæ of yaws as well as in those infected with the spirochetæ of syphilis.

4. This lesion makes possible the investigation of the problems of cultivation, of immunity and of treatment.

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EXPLANATION OF PLATES.

PLATE LIII.

FIG. 1. Colored soldier with yaws; second eruption.

PLATE LIV.

FIG. 2. Monkey 1, ulcerating yaw on eyebrow and eyelid, 47 days after inoculation from patient.

FIG. 3. *Treponema pertenui* from Rabbit A, Giemsa's stain, × 1,000.

FIG. 4. *Treponema pertenui* in rabbit's testicle, Levaditi stain, × 1,500.

FIG. 1.

FIG. 2.

FIG. 3.

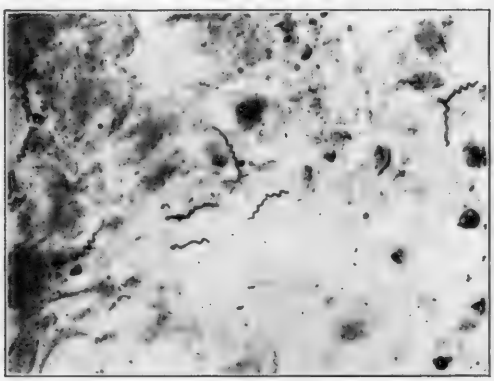

FIG. 4.

CHANGES IN THE HEMOSIDERIN CONTENT OF THE RABBIT'S LIVER DURING AUTOLYSIS.[1]

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The investigation of the changes in the hemosiderin content of the rabbit's liver during autolysis was undertaken with the hope that such an investigation might shed some light on the vexed problems of the mode of formation of the hematogenous pigments, their relation to one another and to hemoglobin, and their ultimate disposition. The work has yielded some very definite results which will be described in the present paper. The author feels that the study of hemosiderin and related pigments under autolytic conditions presents so many technical difficulties and so many sources of possible error as to render a report of the more minute details inadvisable at the present time. For this reason, the scope of this investigation, and of this report, has been confined to those changes which are most evident, and where comparative estimates are based on changes so manifest as to preclude all possibility of error.

It is well to note that the term hemosiderin is here used, as originally applied by Neumann,[2] to indicate the yellow or yellowish brown, hematogenous pigment which reacts for iron with potassium-ferrocyanide and hydrochloric acid or with ammonium sulphide.

The rabbits used for this work may be grouped in four classes: (1) normal (for laboratory animals); (2) infected animals; (3) animals injected with saponin; (4) miscellaneous animals used in the laboratory for other purposes.

TECHNIQUE.

Both control and autolysed tissues have been fixed in 95 per cent. alcohol and in 10 per cent. formalin, as a routine. These have been supplemented by other fixatives, in some instances.

[1] Received for publication May 31, 1910.

[2] Neumann, *Virchow's Arch.*, 1888, cxi, 25.

Autolysis.—Both toluol and chloroform have been employed as preservatives in separate series. Autolysis has been conducted on two general plans.

1. Autolysis in moist chamber with toluol or chloroform as preservative. The liver has been cut into strips 6 to 8 mm. in thickness (the larger the piece that can be kept sterile the better), and placed in large air-tight Stender dishes. The atmosphere of the dish is renewed by frequently removing the cover, and moisture is maintained by moist cotton or water in evaporating dishes.

2. Autolysis in liquids. Salt solution (0.85 per cent.) and distilled water have been employed. Autolysis has been attempted by placing tissues directly into chloroform and toluol. This was done solely as a check upon the effects of these agents upon the tissues.

Autolysis has been carried out at room temperature (20° to 22° C.) and at 37° C. for periods of time up to 17 days.

Sections.—Paraffin and celloidin have been used for sections. In all cases sections stained with hematoxylin and eosin have been compared with unstained mounts and with sections tested for iron. Other procedures have been employed occasionally in attempts to clear up the nature of certain substances.

Determination of Iron.—(1) Ferrocyanide or ferricyanide of potassium (2 per cent. aqueous solution) and 1 per cent. aqueous hydrochloric acid, employed together in equal volumes or used separately. 1 per cent. acid alcohol has been used in a few instances as a check. (2) Ammonium sulphide, diluted one-half.

So much has been said of micro-chemical methods for demonstrating iron that I feel that any attempt to explain my choice of these methods would only add to the existing confusion. The one point I wish to emphasize is the necessity of performing the reaction on the slide and observing the development of the reaction under the microscope. If this precaution is not observed, many important features of the reaction may be entirely overlooked. The time allowed for the end reaction has in no case exceeded one hour, where comparative estimates were concerned.

HEMOSIDERIN CONTENT AND IRON REACTIONS OF NORMAL RABBIT'S LIVER.

The liver of the average laboratory animal varies so widely that it is difficult to establish an absolute normal for either cellular picture or pigment content. From the study of a large number of livers, I find that many livers show some degree of fatty degeneration. The cells are prone to be coarsely granular or "foamy" in character, and practically all livers show some granules of yellowish, or greenish brown hemosiderin as well as similar granules which give no iron reaction, presumably bile pigments. It is not uncommon to find the hemosiderin in the form of clear, refractile granules with a pigmented periphery. The hemosiderin frequently stains slightly with hematoxylin or with eosin, producing almost

black granules in the one case, and copper colored granules in the other. Hemosiderin that stains with hematoxylin usually reacts more quickly and more intensely for iron than that staining with eosin. The type of iron reaction, as here indicated, is quite variable. Using the ferrocyanide method, one may obtain all shades of blue from an exceedingly dark blue to a very light or pale blue. Kupfer's cells and the cells in the liver capillaries occasionally show hemosiderin granules. The amount of hemosiderin varies within narrow limits in different portions of the same liver. No other elements regularly react for iron with the technique here employed. Exceptionally, however, some liver cells show a few colorless or faintly pigmented granules in the nucleus which stain with hematoxylin or, more rarely, with eosin, and which give a decided iron reaction. Occasionally, the entire nucleus reacts for iron, and the cell protoplasm may give a faint, diffuse reaction. Likewise, the connective tissue, particularly about portal vessels, and the vessel walls may give the most intense iron reaction. All of these reactions are more marked and more frequent after alcohol than after formalin fixation, and when tissues are not fixed immediately on the death of the animal. None of these features can be considered strictly normal.

One other feature deserves special mention and this is not peculiar to the rabbit. All tissues fixed in alcohol show a peripheral zone of condensation. This entire zone, in the rabbit's liver, both tissue elements and vascular contents, takes a copper-colored stain with eosin and gives a definite reaction for iron. Outside of the tissue proper is a layer of coagulum showing the above character and containing a number of irregular black or greenish brown, refractile, pigmented masses. In the rabbit, patches of greenish yellow are not uncommon and may extend into the zone of condensation. Unstained sections show these zones to be truly pigmented, containing both granular pigment and a diffuse pigmentation of tissue elements and vascular contents. These are features which have undoubtedly been noted by many but no one seems to have attached any importance to them.

In brief then, a section of liver from an average animal, fixed in alcohol, will show:

1. A peripheral zone of pigmented material reacting for iron and containing granular, pigmented masses and patches of pigment which do not react for iron.

2. A zone of condensation with granular and diffuse pigmentation of all elements which react decidedly for iron as well as granular pigment which does not react.

3. The depths of the section show a few granules of hemosiderin and a few granules of bile pigment in the liver cells, the cells of Kupfer, and the leucocytes.

4. The tissue elements do not react for iron.

DELAYED FIXATION.

As a preliminary to the study of the effects of true autolysis, some experiments were undertaken to determine the changes in the hemosiderin content of the liver after the fixation of large pieces of liver or whole livers in alcohol of various strengths (50 per cent. to 95 per cent.). Under such conditions fixation is delayed, in some cases for from three to four days where whole livers and weak alcohols are used. The center of such tissues shows changes comparable, in some respects, to autolysis. The changes in this series of experiments are described as the effects of delayed fixation.

The change from the normal picture in delayed fixation is quite striking in many cases and may best be illustrated by a typical experiment.

H. R. E.—Normal rabbit, male, albino, weight 2,500 grms. Killed by blow on head. Small pieces of liver fixed in 95 per cent. alcohol and 10 per cent. formalin for control. One of the largest lobes placed in a small amount of 95 per cent. alcohol at 37° C.

Control.—The control (alcohol fixed) corresponds closely with the above description of the normal liver though slightly more fatty.

Twenty-one hours at 37° C.—Section taken which extends through entire thickness of the lobe. The central portion is not yet fixed. The liver cells stain more decidedly with eosin and the protoplasm is more granular. Most of the granules show a slight affinity for hematoxylin. The pigment granules of the liver cells average twice that of the control. The peripheral and condensation zones appear on the two capsular surfaces and correspond closely with the control. Central from the zone of condensation, about 2 mm., there is an irregular band, parallel to the surface, several millimeters in breadth, in which the cells show a slight, copper colored staining. The granular pigment in this area is strikingly increased, averaging 4 to 6 times that of the control.

Unstained sections show peripheral and condensation zones comparable to the control. There is a band of faint, yellowish, diffuse pigmentation just central from the zone of condensation. This also shows marked increase in the granular pigment of the cellular elements. The granular pigment is increased throughout the section to an average of double the control.

Iron Reactions.—Grossly, there is a broad band of marked reaction on the two capsular surfaces. Central from this, there is a second band of slightly less decided reaction, while the entire section shows a heavier reaction than the control. Microscopically, the peripheral and condensation zones show a reaction about parallel with the control. The second band, above mentioned, shows a marked reaction in the cell protoplasm, though more pronounced in the nuclei. There is also a marked increase in the granular pigment reacting for iron. Cell nuclei in the depths of the section show a faint iron reaction with intranuclear granules which react sharply. The cell protoplasm reacts very slightly. Pigment granules (hemosiderin) are increased throughout. The connective tissue throughout the section also reacts faintly for iron.

Forty-eight hours at 37° C.—The section taken as above. Fixation is complete. The features of the 21-hour stage are but very slightly altered. The iron reaction in the central portion is slightly more pronounced and the hemosiderin slightly increased.

This experiment shows the lesser changes which have prevailed with normal animals and those showing minor degrees of fatty degeneration in the liver. Animals with infections, such as staphylococcus, and animals injected with saponin to produce destruction of red blood corpuscles and increased pigment production, present the same general changes, though to a much more marked degree. This is illustrated by the following case.

H. R. I. Gray and white, weight 1,900 grms. Between Jan. 7 and 24, the animal received seven injections of saponin in the ear vein, amounting to 19 mg. The animal died soon after the last injection. Autopsy was performed one hour after death. Tissues were treated as in H. R. E.

Control.—Liver shows foci of necrosis and hemorrhage with marked congestion. The liver cells contain numerous dark brown granules of hemosiderin, grouped particularly about the nuclei. The nuclei are large and vesicular with many slightly pigmented basophilic granules which react for iron. The foci of necrosis and hemorrhage show a faint diffuse iron reaction in all structures and slightly more granular pigment than other portions of the section. The connective tissue throughout reacts faintly for iron. The zones of condensation and peripheral coagulum react strongly for iron.

Twenty-one hours at 37° C., in 95 per cent. alcohol.—Section taken as with H. R. E.; center is unfixed. Cell nuclei are large and vesicular. The granular bodies of the nucleus have largely disappeared. The nuclei and remaining granules react strongly for iron. The hemosiderin of the liver cells is increased, on an average, 4 fold. The cell protoplasm and connective tissue throughout react strongly for iron. While there is a marked increase in iron

reacting material of the capillaries and deeper vessels, those nearer the capsular surfaces (exposed surfaces) show the most marked changes. In these areas the entire red blood corpuscle content is fused into large and small globular and angular masses, many of which are grouped into clusters with clear refractile bodies at the center. Most of these bodies show a darkly pigmented periphery and are identical with granules of hemosiderin in the section. When unstained, all such masses are yellowish brown in color. They stain a coppery red with eosin. The entire mass reacts strongly for iron, perhaps the central crystalline body most intensely. The capsular surfaces show the two zones of the control section and a third zone which is identical in character with that described for H. R. E. This zone shows intense pigmentation, diffuse and granular, and the iron reaction is so intense as almost to obscure the structural elements.

Ninety-six hours at 37° C., in 95 cent. alcohol.—This stage shows but little change from the 21-hour stage. The granular hemosiderin is slightly increased and Zone "3" is broader. The iron reaction is intensified in all particulars.

While these two examples, taken from eighteen such experiments, represent, in type, the usual findings, such changes have not always been so marked, nor have I found them constantly. In a few instances, I have obtained a reduction in the hemosiderin content, but this has been accompanied by the appearance of a marked nuclear reaction for iron and, occasionally, by a diffuse reaction of liver cells and connective tissue similar to that just described.

The changes which should be particularly noted in delayed fixation are as follows: (1) increase in the hemosiderin content of the liver cells; (2) formation of hemosiderin in large amounts directly from the red blood corpuscle contents of vessels; (3) development of a diffuse pigmentation and iron reaction in the cellular elements; (4) development or intensification of a nuclear reaction for iron; (5) development of a zone beneath all exposed surfaces in which all changes are most pronounced and which might be termed the "zone of reaction."

AUTOLYSIS.

For maintaining antiseptic conditions during autolysis, toluol is to be preferred to chloroform. Though the latter is more efficient in preventing bacterial growth, it is very apt to fix the tissues and prevent autolysis. Pieces of tissue placed directly in chloroform are fixed slowly with marked shrinkage and distortion of cellular elements. All red blood corpuscles are laked and the hemoglobin

coagulated on the surface in irregular globular masses which are yellow or brownish in color. A large part of such masses manifests a faint though definite iron reaction. Other changes, comparable to those noted under delayed fixation, are observed, though all such changes are on the surface or in superficial parts of the tissue. The pigment content in the depths of the section is usually reduced.

Of the methods of autolysis which I have employed, the moist chamber with toluol as preservative has given the most constant and trustworthy results. Submersion in liquids, except perhaps toluol, is not permissible, as some products formed during autolysis are soluble in such liquids and are lost from the section. In order not to burden this report with too many details of the different stages of autolysis, I will give the essential facts deduced from this entire series of experiments.

A definite increase in the hemosiderin content of the liver has been noted as early as the fifth hour, but, as a rule, the change is not marked before the twelfth or even the twenty-fourth hour. After such an interval, the exposed surfaces of the liver have become brown in color, while the side upon which the strip rests is of its original color or paler. Cross section shows this pigmentation extending two to four millimeters into the substance of the tissue. If the strip is turned frequently during autolysis all surfaces will show this change. Examination of the material oozing out of the liver, shows large and small masses of both colorless, refractile material, and pigmented material, reacting for iron, as well as numbers of hematoidin crystals. Sections, at this stage, show on the surface, variable amounts of angular and globular pigmented masses reacting for iron. There is a narrow zone of condensation (alcohol fixed tissue) which usually reacts only faintly for iron, and a third "zone of reaction" similar to that described with delayed fixation. This zone is much more pronounced in autolysis and, in sections tested for iron, it stands out grossly, as a band two to four millimeters in breadth about all surfaces exposed to the air during autolysis, while the unexposed surface shows little or no trace of such a zone.

In the depths, the granular hemosiderin in the liver cells is in-

creased, on an average, two fold, and there is a corresponding increase in iron-containing pigment in the capillaries and larger vessels. Stained sections show that as the nuclei undergo autolysis the hemosiderin and granular material of tissues and vessels take a more or less decided hematoxylin stain, as might be expected. The granules in cell nuclei, and occasionally entire nuclei, react for iron. All other tissue elements react with varying intensity. These changes in iron reaction are nearly all referable to varying degrees of pigmentation shown in unstained sections. The maximum change of this character has usually been reached before forty-eight hours at 37° C. Occasionally, the maximum was shown at seventy-two hours, 37° C.

After the maximum of pigmentation and iron reaction has been reached, a second series of changes gradually becomes predominant. These changes are first noted in the depths of the tissues and are coincident with the dissolution of the cell nuclei, succeeding the stage noted above when the hemosiderin and other granules show definite hematoxylin staining. The hemosiderin granules gradually become brighter in color and more decidedly yellow, both in stained and unstained sections. The iron reaction in pigment granules gradually decreases and ultimately fails entirely. There is no apparent reduction in the amount of such granular pigment, and in some cases there seems to be an increase. Irregular, pigmented masses become more numerous in the capillaries between the columns of liver cells. Large amorphous and crystalline masses of both colorless and dark greenish brown material collect irregularly through the section and particularly in and about the vessels and bile ducts of the portal spaces. The material in the portal spaces is practically always pigmented. The crystalline masses occur in many forms, irregular plates, rosettes or clusters of needles or rhombic plates, and large feathery crystals. The latter are usually colorless, exceedingly hard and glass-like, and it is almost impossible to obtain good sections where such are abundant. The colorless masses and crystals stain a pale slaty blue with hematoxylin, while the pigmented forms appear unaffected by stains.

Such pigmented material is slightly soluble in water, more so

in alcohol and is quickly dissolved or destroyed by dilute mineral acids or by alkalies. The solution in any case, unless it be with alkalies, is not complete as there always appears a dark or yellowish brown residue when the process is observed under the microscope. In a few instances, where one per cent. aqueous hydrochloric acid was employed, fine crystals of hematoidin appeared about the site of the original mass. The solubility of the colorless forms has been more difficult to follow, though it is, apparently, similar to that of the pigmented forms.

A large part, and perhaps all, of these masses react for iron and for phosphorus by the method of Lilienfeld and Monti.[3] If the Prussian blue method is employed in testing for iron; by following the reaction under the microscope, the amorphous or crystalline material will be observed to disappear with the production of a diffuse flocculent precipitate of Prussian blue about its former site. Portions of such masses, or an occasional crystal, may react without dissolution, in which case the Prussian blue is formed *in situ*. Material, apparently identical with that described above, occurs in abundance as a gritty, crystalline deposit in the *brei* in the dish used for autolysis. When tissues are autolyzed in liquids, no such crystals and amorphous masses are found in the sections, nor to any extent in the liquids.

The series of changes initiated by the disappearance of the iron reaction from the hemosiderin of the liver cells in the depths of the tissue, and culminating in the formation of amorphous and crystalline masses containing both iron and phosphorus, is in progress for several days. During this period, such changes approach the more superficial parts where pigment production and iron reaction are most pronounced. This zone resists such transformation for a variable length of time, and, while the change ultimately becomes marked, it is never so complete as in the other parts of the section, as far as my experiments have extended (seventeen days at 37° C.).

Returning to the yellowish granular pigment in the liver cells, which no longer reacts for iron, I may say that it has been impossible for me to determine with certainty what this pigment is. The color is slowly and incompletely extractable with alcohol and with

[3] Tracy, *Jour. of Med. Research*, 1906, xiv, 447.

chloroform, apparently more readily soluble in alcohol than chloroform, but with difficulty in either. Alkalies and mineral acids either extract or destroy the color. After the extraction of the color of these granules I am convinced that the body of the granules still persists as an insoluble residue. From the above facts I believe that the pigment in question belongs to the class of bile pigments.

I have found it impossible to exercise a check on the changes in the bile pigment content of the liver cells with any degree of accuracy. The use of mercuric chloride as a fixative has been recommended, but I have found it of little value. The occurrence of hematoidin crystals in the *brei* and the solution of bile pigments in the alcohol used as a fixative have served as rough guides. In the *brei,* hematoidin crystals usually appear within ten to twelve hours and gradually increase in number for two to three days, after which they decrease, and are perhaps replaced by other oxidation products. In stages as late as fifteen days, I have found them practically absent.

In studying the *brei* for hematoidin crystals, I became impressed by the fact that they were almost always attached to or imbedded in slightly pigmented or, more often, perfectly colorless, refractile masses along with a few small masses of hematin. Such a matrix always reacts most intensely for iron and is incomparably greater in amount than either, or both, the hematoidin and the hematin. The hematin apparently develops after the addition of dilute acid to clear the preparation or in applying the test for iron. Hematin has never been found in any considerable amount in any of my preparations except in sections and tissues kept for some time in alcohol.

This raises a point in technique which is of utmost importance, namely, that the tissues should be sectioned and studied at once, without being allowed to stand in alcohol. Tissues or sections kept in alcohol change very much in a short time. The three changes of most importance which I have noted are: (1) solution of certain products; (2) decrease and marked alteration in the type of the iron reaction coincident with; (3) the appearance of enormous amounts of hematin throughout the section.

In one instance a pronounced iron reaction was present in the nuclei and diffusely through other elements when tested immediately after fixation. One month later, section left in 80 per cent. alcohol showed practically no iron reaction, but, instead, granules of hematin were found in practically every nucleus and diffusely throughout the sections. Similar changes, though less marked, have been noted in a number of instances.

In recasting the results obtained in this investigation, certain features stand out prominently. First, in both the experiments with delayed fixation and with autolysis, there is a definite increase in the granular hemosiderin of the liver cells and in the formation of masses of hemosiderin in the capillaries and larger vessels as well as in the diffuse type of pigmentation. Second, coincident with this increase in hemosiderin during autolysis, or, more likely, following closely upon such increase, there is an increase in the hematoidin in the *brei,* and the development in the liver cells of granules of pigment which do not react for iron, but are partially soluble in alcohol and in chloroform; hematoidin and other bile pigments are demonstrable in such solutions. Finally, the hemosiderin of the liver cells loses its iron reaction as the nuclei undergo dissolution, and large amounts of colorless and pigmented, amorphous and crystalline material, containing both phosphorus and iron, appear throughout the section, but more particularly in the portal spaces.

These findings show, most conclusively, that hemosiderin may be formed in organs outside the animal body, such formation continuing, under the conditions herein employed, for twenty-four to forty-eight hours after the death of the animal. The conversion of large amounts of hemoglobin *en masse* in the vessels shows that hemosiderin can be formed directly from hemoglobin.

The evidence as to the character of the process and the factors concerned is not entirely conclusive. The fact that such changes take place best under conditions favoring enzyme action suggests the possibility of such an agent. The action of the agent in question is not confined to the tissue cells but is equally active on the contents of relatively large vessels and on hemoglobin on the surfaces of tissues as well as in the *brei* in the moist chamber. The invariable predominance of pigment production on and in surfaces

exposed to the air deserves especial emphasis, as it suggests most strongly that oxygen is a potent factor in hemosiderin production.

The idea that hemosiderin can be produced *post mortem,* or can be formed except by the activity of a living cell, is opposed to present teachings.[4] The activity of an oxidizing enzyme has been suggested by Neumann.[5] So far as I am aware, all theories that have been advanced to explain hemosiderin production have considered hemosiderin as a derivative of hematin. If it is intended to convey the idea that hemosiderin is derived from the hematin moiety of hemoglobin, without the necessity of hematin being formed as an essential step in hemosiderin production I can offer no objection to such theories on this ground. On the other hand, the idea that hematin is a necessary antecedent of hemosiderin is untenable. I have carefully traced the formation of hemosiderin from hemoglobin through all stages of autolysis and have observed nothing that would lead me to suspect that the production of hematin was in any way essential. Further than this, hemosiderin is a proteid substance while hematin is the non-proteid or pigment radicle of hemoglobin, a cleavage product. Hemosiderin can also be shown to be capable of cleavage into hematin and a proteid by the action of acids as dilute as 5 per cent. sulphuric. This can be applied to sections and watched under the microscope.

The relation of hematoidin to hemosiderin is not entirely clear. It seems to be formed in greatest abundance subsequent to the formation of hemosiderin. The strongest evidence of the relationship of these two pigments has been derived from the phenomena described in the *brei* of autolyzing tissues. It will be recalled that I mentioned the occurrence of large, faintly pigmented, or colorless, hyaline masses, reacting strongly for iron, attached to or imbedded in which were numerous hematoidin crystals. Such pictures may have been chance findings but they have been exceedingly frequent. Virchow,[6] in his classical monograph, both described and pictured this relationship essentially as I have observed it, except for the iron reaction. While he refers to hematin as the mother substance, it must be borne in mind that the terms "hemoglobin" and "hemo-

[4] Neumann, *Virchow's Arch.,* 1904, clxxvii, 401.
[5] Neumann, *loc. cit.*
[6] Virchow, *Virchow's Arch.,* 1847, i, 379.

siderin" had not been introduced at that time, and he must have referred to the coloring matter of the red blood corpuscles. This picture represents my views at the present time of the relationship of hemosiderin and hematoidin, namely, that hematoidin is the pigment radicle of hemosiderin. When the hematoidin is removed, the residue is a colorless, refractile substance, reacting for iron; or, stated differently, hemosiderin is a colorless iron-containing proteid plus hematoidin.

Before passing to the final series of events, I wish to consider briefly those anomalous iron reactions occurring in nuclei, cell protoplasm, and connective tissue, which as I have mentioned are occasional findings at death, and develop during delayed fixation, and in the early stages of autolysis. Such phenomena occurring under the last two conditions are capable of at least three interpretations: (1) changes in the cell constituents capable of rendering masked iron demonstrable; (2) the formation from hemoglobin of a substance capable of manifesting an iron reaction; (3) the decomposition or diffusion of some iron-containing substance similar to hemosiderin.

The first of these explanations seems the least likely of the three. It is wholly inadequate to explain those cases which occur at death. Such reactions occur in animals in which blood destruction and hemosiderin formation are above normal. Granular, pigmented bodies are occasionally demonstrable in such nuclei. These granules stain with greater or less intensity with hematoxylin, but frequently show a refractile, unstained center. The iron reaction is usually confined to these granules, though the entire chromatin network may react. Further, the hemosiderin is closely packed about the nucleus and can be found penetrating the nuclear membrane. The explanation of the nuclear reaction, in these cases, is undoubtedly referable to the hemosiderin and, as I believe, to its decomposition rather than to its production, though both are possible factors. The reaction of other elements is more likely due to excessive production of hemosiderin.

The development of an iron reaction in nuclei during autolysis is undoubtedly due in part to hemosiderin production, as the reaction is shared by all structures. Further, the formation of hematin

in such nuclei, and its diffuse distribution through the tissues after keeping sections in alcohol, coincident with the disappearance of the iron reaction, indicate that hemosiderin is an important factor in such iron reactions. It must be admitted, however, that it is impossible to prove that autolysis has not rendered demonstrable the masked iron of such tissues, though I hardly believe the autolytic changes, occurring in the alcohol experiments, can be sufficient to account for the iron reactions obtained in this series.

From the explanation of these peculiar iron reactions, and from changes noted in the exposed portions of tissues during autolysis, we find an explanation for the iron reacting coagulum and zone of condensation of normal liver fixed in alcohol. While it is impossible to explain either of these solely on the theory of solution or diffusion of hemosiderin, or contraction *per se,* and while both of these factors may be accessory, there is certainly an actual conversion of hemoglobin into hemosiderin in these areas.

The final series of changes concerned with the disappearance of the iron reaction from the hemosiderin and the formation of amorphous and crystalline material containing both phosphorus and iron, is of especial interest. While it is impossible to exclude other factors absolutely, the circumstances under which these compounds are formed indicate strongly some interaction between the nuclear phosphorus and the iron of the hemosiderin. It is quite certain that the iron of the hemosiderin is split off coincidently with nuclear dissolution which probably supplies the phosphorus. Further than this we can not go. The exact nature of the compound in question has not been investigated. Bile pigments are an admixture in some cases. The residue of the hemosiderin, after the cleavage of the iron, also contains extractable bile pigments.

This peculiar series of phenomena, taken in conjunction with the nuclear picture in animals where hemosiderin production and metabolism are excessive, furnishes an interesting clue to the vital metabolism of hemosiderin in the liver and the fixation of its iron content. Hemosiderin seems to be formed as an oxidation product of hemoglobin, and in its further metabolism an acid radicle of the nucleoproteid binds the iron, while the coloring matter, hematoidin, is excreted as the bile pigment.

Changes analogous to those described for the liver have been observed in the kidney, spleen and mesenteric lymph nodes of the rabbit, though they have not been studied to any considerable extent.

Further experiments upon the relation of oxygen to hemosiderin production and the effect of organ extracts upon hemoglobin are in progress. Results, thus far obtained, seem to confirm the results of the present investigation.

CONCLUSIONS.

1. Hemosiderin may be produced outside of the animal body and is increased in the liver of rabbits, during autolysis, for a period of from twenty-four to forty-eight hours.

2. Post-mortem hemosiderin formation is most marked in parts of livers exposed to air, and hemosiderin is, apparently, an oxidation product of hemoglobin due to enzyme action.

3. Hemosiderin is derived from hemoglobin directly and not from hematin as an intermediate product.

4. The later stages of autolytic changes show that the acid products of proteid autolysis, especially the phosphorus acids, are capable of producing a cleavage of hemosiderin and uniting with the iron to form a new series of products which react microchemically for both phosphorus and iron. In such cleavage, pigments analogous to the bile pigments are formed.

5. The relationship observed between hemosiderin and hematoidin is such as would indicate that hematoidin is the pigment matter of hemosiderin.

6. Further, it seems probable that the vital cycle of hemoglobin metabolism in the liver is largely intranuclear; the hemoglobin is converted into hemosiderin either within the nucleus or cell protoplasm; the iron of the hemosiderin is bound by an acid radicle of the nucleo-proteid, and the hematoidin is excreted as bile pigment.

THE EFFECT OF VAGUS SECTION UPON ANAPHYLAXIS IN GUINEA PIGS.[1]

SECOND COMMUNICATION.

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PLATES LV AND LVI.

INTRODUCTION.

In a previous paper[2] dealing with some of the functional disturbances of a type of very acute serum anaphylaxis in the guinea pig, we came to the conclusion that the death of the animal was due to asphyxia caused by a tetanic contraction of the bronchial muscles, the contraction being so pronounced that the lumina of the smaller bronchial tubes were occluded, thus preventing both the entrance and escape of air. This tetanic contraction imprisoned the air in the lung so that this organ could be excised *in toto* with practically no collapse. The evidence for the conclusion stated above was fully considered in this earlier paper[3] and I shall not enter upon this question now; it may, however, be added that this view has since been shared by Anderson and Schultz[4] and by Biedl and Kraus.[5]

On the basis of this assumption, therefore, it became of interest to determine what the effect would be if the bronchial musculature were deprived of its innervation. In this way, perhaps, it could be established whether the toxic injection of serum affects the nerve endings in the bronchial muscles, or the muscle substance directly,

[1] Received for publication June 24, 1910. A preliminary note was published in the *Proc. of the Soc. for Exper. Biol. and Med.*, 1910, vii, 103.

[2] Auer and Lewis, *Jour. of the American Med. Assn.*, 1909, liii, 458; *Jour. of Exper. Med.*, 1910, xii, 169.

[3] Auer and Lewis, *Jour. of Exper. Med.*, 1910, xii, 163–169.

[4] Anderson and Schultz, *Proc. of the Soc. for Exper. Biol. and Med.*, 1910, vii, 34.

[5] Biedl and Kraus, *Wiener klin. Woch.*, 1910, xxiii, 385.

or both structures. Now it is well-known that the vagus nerve supplies the motor innervation for the bronchial muscles;[6] section of this nerve, therefore, and an adequate lapse of time for degeneration of the peripheral stump would rob the bronchial muscle of its nerve supply, provided that the vagus motor fibers are not preganglionic. This method, therefore, would seem to permit an experimental approach to the problem.

EXPERIMENTS.

The experiments were carried out entirely in guinea pigs, and the members of each series always belonged to the same lot and were approximately of the same age and weight. They were treated exactly alike in the preparation; the same horse serum in the same amount, either one or two cubic centimeters, was injected subcutaneously for sensitization; the vagus nerve was resected in the neck under ether anesthesia, and it was attempted to remove the left or right vagus in an equal number of guinea pigs. After a varying period of time (details will be given later), the toxic dose was always injected into the jugular vein through a canula, the injection being followed by one cubic centimeter of Ringer solution to wash out the canula. For the toxic dose I invariably employed horse serum which had been heated to 55° C. for thirty to thirty-five minutes. The toxic injection was, as a rule, just above the surely fatal dose.

Before entering upon a report of the experimental results, some suppositions, basic for this investigation, must be discussed. From the arrangement of the experiments stated above, it will be seen that two fundamental assumptions are made, which are of vital importance. These assumptions are that the bronchomotor fibers of the vagus do not end in ganglion cell stations, are not preganglionic, in other words; and, secondly, that these motor fibers do not decussate, so that one vagus innervates only the lung on the same side.

For the first assumption, that the bronchomotor fibers of the vagus are not preganglionic, there is sufficient evidence. It is well known that the motor fibers for the bronchial muscles in the neck

[6] For literature see Dixon and Brodie, *Jour. of Physiol.*, 1903, xxix, 115.

vagus remain irritable for a comparatively long time after the death of the animal.[7] Now, if the motor lung fibers in the vagus were preganglionic, this ought not to be possible, for Langendorff[8] showed that stimulation of *preganglionic* fibers swiftly becomes ineffective after death of the animal by bleeding or by asphyxia, while stimulation of the *postganglionic* fibers will then still yield results. Langendorff's results have been corroborated and extended by Langley[9] who states that postganglionic fibers give the customary response one quarter to three quarters of an hour after death, while preganglionic fibers soon lose their effectiveness after death. Langley also states that he has found the same thing true in all sympathetic fibers with which he has experimented.

That the guinea pig forms no exception to the general rule, I have had some occasion to observe accidentally and in experiments made especially for this purpose. Occasionally, an animal died through some accident or was killed after having been prepared for registration of intrapleural pressure or of lung volume by oncometers. In these animals stimulation of the vagi still showed distinct and definite changes in the intrathoracic pressure or of lung volumes fifteen and twenty minutes after the heart had ceased to beat (Plate LV, Fig. 2, Plate LVI, Fig. 3). From the data submitted, it will, therefore, be seen that there is good evidence for the assumption that there is no cell station between the bronchomotor fibers of the neck vagus and the bronchial muscles themselves.

In this connection it may be observed that no very marked differences between the two vagi, as far as the bronchomotor effect is concerned, were noticed. This is in agreement with the observation of Einthoven,[10] though other investigators have found that the left vagus usually exerts a greater effect than the right.[11] Occasionally I have observed animals in which stimulation of one vagus gave only slight results, while the other vagus responded well. This diminished irritability occurred sometimes in the right, sometimes in the left vagus.

The second assumption was that the bronchomotor fibers of the two vagi do not decussate, that thus each vagus innervates only its

[7] For references see Dixon and Brodie, *loc. cit.*, 115, 152.
[8] Langendorff, *Cent. f. Physiol.*, 1892, v, 130.
[9] Langley, *Jour. of Physiol.*, 1893, xv, 181.
[10] Einthoven, *Pflügers Arch.*, 1892, li, 381.
[11] For literature see Dixon and Brodie, *loc. cit.*, 118–119.

ipselateral lung. Dixon and Brodie experimented on cats, dogs and rabbits and they state explicitly that both the constrictor and dilator fibers in the vagus supply the lung on the same side only.[12] As there were no observations on the guinea pig, I made some experiments to test this question, though it seemed improbable that the guinea pig should form an exception. The results confirm largely the statement of Dixon and Brodie; in only one or two experiments did I find that stimulation of one vagus occasionally exerted a bronchoconstrictor effect upon both lungs (Fig. 2), and in one of these experiments it is noted that the vagus was stimulated low down so that the possibility of current escape to the other vagus must be taken into consideration. Fig. 3 gives a qualitative picture of the usual result obtained. If there is a decussation of vagus bronchomotor fibers, it cannot be an extensive one, and, therefore, need not be taken into serious consideration.

Another assumption made is that all the bronchomotor fibers run in the vagus. I have no experimental data of my own in support of this, but Dixon and Brodie[13] have found that the sympathetic in cats, dogs and rabbits carries no bronchodilator or bronchoconstrictor fibers, and it is not likely that the guinea pig forms an exception to this.

The considerations given above show, I think, that the assumptions made for this investigation are reasonably grounded on experimental facts.

RESULTS.

In the *first series* of experiments both vagi were cut in sensitized guinea pigs shortly before the intravenous injection of the toxic dose. The course of the immediate anaphylactic reaction was perfectly typical and ended in death within five minutes. The lung picture was characteristic when the thorax was opened and the organ excised.[14] Section of both vagi exerted no appreciable effect in any way on the onset, course and final outcome of the reaction.

In the *second series* nine guinea pigs were used. They were all

[12] Dixon and Brodie, *loc. cit.*, 172.

[13] Dixon and Brodie, *loc. cit.*, 142, 172.

[14] For a full description of the gross appearance of the acute anaphylactic lung, see Auer and Lewis, *Jour. of Exper. Med.*, 1910, xii, 156.

sensitized on the same day by the subcutaneous injection of one cubic centimeter of horse serum. Thirteen days after sensitization, one to two centimeters of one vagus nerve was resected in the neck; in five animals the left vagus was resected, and in four, the right. The toxic dose was injected thirty to fifty-seven days after vagus section; two animals were utilized after thirty days, two after thirty-one days, three after forty days, and the remaining two fifty-seven days after vagus section. The toxic dose ranged between 0.8 cubic centimeter to 1.5 cubic centimeters of a 10 per cent. solution of heated horse-serum. In six of these animals death occurred under typical symptoms within four minutes; for purposes of convenience, death was considered to occur when the respiration had permanently stopped.

The autopsy picture of the lungs was typical. On opening the thoracic cavity by first cutting away the thoracic attachments of the diaphragm, the lungs were seen to fill the entire cavity and appeared to suffer no appreciable collapse. No difference could be seen between the two sides; both appeared equally large. On total removal of the lungs from the thorax no collapse was seen, and more careful comparison showed that both sides, innervated as well as denervated, possessed about equal volume (Plate LV, Fig. 1). The lungs looked, in short, exactly like those obtained from an ordinary sensitized guinea pig which had succumbed to the toxic injection within five minutes. In two instances the left half of the lungs looked a trifle larger and fuller than the right, this being especially true of the lower lobes. In one of these animals, the left vagus has been resected, and in the other, the right. Therefore, vagus section could not be held accountable for this difference in size. Moreover, I have frequently had occasion since the beginning of this work to see that anaphylactic guinea pigs with intact vagi often show a slight difference in fullness between the two sides of the lungs, and that this fullness occurs more frequently in the left, especially the lower lobe, than in the right side.

In three animals of this series sublethal doses of horse serum were injected and the animals then killed by section of the medulla. The lungs of these animals were full and large, but not to the same degree as in the six other animals. There was again no difference between the two sides of the lungs.

In this series, resection of one vagus exerted no visible effect on the gross appearance of the lungs.

In a *third series,* composed of eight animals, the vagus was resected in four on the right side and in four on the left side fifty-five days after sensitization (one cubic centimeter horse serum subcutaneously). The toxic dose was injected intravenously in three animals six days after vagus section, in two after thirteen days and in the remaining three after fourteen days. In the first three animals the toxic dose employed was 0.6 cubic centimeter heated horse serum; in all the rest 0.3 cubic centimeter of a 10 per cent. solution of heated horse serum was used. All the animals except two died with typical symptoms within five minutes, and these two survivors had received, previous to the toxic dose, a prophylactic injection of atropine.

The lungs on autopsy again showed no difference between the two sides which could be ascribed to the vagus section; they looked like typical anaphylactic lungs, like normal lungs which had been fully inflated and fixed in that position. In two cases the left sides of the lungs looked fuller than the right; in one of them the left vagus had been resected, and in the other the right; all the others (four) showed no appreciable difference in fullness between the right and left halves of the lungs.

In this series, also, vagus resection did not exert any influence upon the production of the typical anaphylactic lung.

In a *fourth series* of animals, the left vagus was resected in four animals and the right in three. All these animals were normal and non-sensitized when the operation took place. Sensitization was performed thirty-three days after vagus section by injecting subcutaneously one cubic centimeter of horse serum. The toxic dose was injected intravenously fourteen days later in five animals and fifteen days later in two. The toxic dose varied for the first five animals between 0.3 and 0.5 cubic centimeter; in the remaining two animals 0.15 cubic centimeter was injected. All except one of the animals responded typically to the second injection of horse serum and died within seven minutes (in one instance only was this time consumed). The exception noted above was due to intravenous injections of atropin. Autopsy demonstrated the typical lung pic-

ture of immediate anaphylaxis[15] in guinea pigs. In this series again no characteristic differences between the two sides of the lung could be seen. Three showed no differences at all between the two sides of the lungs (one had the right vagus resected, the other two, the left vagus); two showed the left lower lobe a trifle smaller than the right lower lobe (one animal was deprived of the right vagus, the other, of the left); the remaining animal possessed lungs in which the left lower lobe was larger than the right lower lobe (here the left vagus had been resected). From these data it is again easily seen that no macroscopic changes in size of the lungs were observed with any constancy which could be attributed to interference with the nerve supply.

Atropin.—In a few animals of these series the effect of atropin was studied for the following reason: according to the prevailing conception of the action of atropin, this alkaloid in moderate doses acts only on the nerve endings and not on the muscles. On the assumption (which did not appear very likely to me) that the sensitization might affect largely the nerve endings, I argued that a prophylactic injection of atropin[16] might show a striking picture if the animal were killed, by cutting the cord, for instance, some minutes after the toxic injection; the innervated lung ought to be collapsed while the denervated lung side ought to stand up fully distended with air when the thoracic cavity was opened, if the sensitization affected the nerve endings. The experimental test, however, did not yield this result. Three out of five animals which received subcutaneously a prophylactic injection of 3 mg. of atropin survived the toxic injection and were killed 12 to 15 minutes later by cutting the cord; it may be added that five controls all died within five minutes. Immediate autopsy showed that the lungs collapsed well as soon as the chest cavity was opened, and that there was no appreciable difference between the two sides. The atropin had affected both sides equally, innervated as well as denervated. It may be argued that 3 mg. of atropin for a guinea pig weighing approximately 300 grm. is not a small or even moderate dose, for, in proportion, an individual of 60 kg. would receive more than 500 mg. of this powerful alkaloid. While this method of obtaining comparable proportionate doses in different species of animals by utilizing their weight only is not entirely sound, because it ignores too many other factors which have an important influence in determining the proper dose, yet it must be concluded that the dose of atropin given to these guinea pigs was large enough to affect the bronchial muscles directly. It would have been

[15] By "immediate anaphylactic reaction" we designated a type of acute anaphylaxis in which the animal usually dies within five minutes after the intravenous injection of the toxic dose. See Auer and Lewis, *Jour. of Exper. Med.*, 1910, xii, 153, 172.

[16] Auer and Lewis, *Jour. of the American Med. Assn.*, 1909, liii, 458. This subject will be more fully considered in the third communication.

useless to test the effect of a smaller dose of atropin, for, as stated above, 3 mg. failed to save two of the guinea pigs from death. This atropin series of experiments, therefore, gave no information about the function of the nerve endings in anaphylaxis.

DISCUSSION.

The results obtained in the various series of animals with one resected vagus all clearly show that an intact vagus innervation of the bronchial muscles is not necessary for the production of the typical lung picture of immediate anaphylaxis. This is especially well shown in the fourth series of experiments where the vagus was resected and allowed to degenerate before the animal was sensitized. In this series sensitization probably occurred after the vagus had completely lost its effect on the bronchial muscles,[17] for the sensitizing dose of serum was injected thirty-three days after the vagus had been resected, and a time period of almost five weeks is, in all likelihood, sufficient for complete functional degeneration of the distal segment of the severed vagus. Moreover, it must be noted that the toxic dose of horse serum was injected after what is practically the minimum length of time necessary for a certain sensitization, that is, after fourteen days. Yet these animals with one vagus resected were as fully sensitized as normal guinea pigs would be after the same sensitizing dose. There was no sign of a slowed or decreased sensitization; on the contrary, it seemed to me as if these animals were perhaps more highly sensitized than normal animals treated with the same sensitizing dose and used after the same lapse of time; these guinea pigs, with one vagus resected, responded promptly and characteristically with as little as 0.3 and 0.15 cubic centimeter of heated horse serum. Be this as it may, there was surely no retardation or reduction in the sensitization caused by robbing the bronchial muscles of their nerve control. It may, therefore, be legitimately concluded that the bronchial muscles themselves, or at least those of the finer tubes, may be sensitized by horse serum after the vagus innervation of these muscles has degenerated.

[17] Dixon and Brodie, *loc. cit.*, p. 141. These authors found that 14 days after section stimulation of the peripheral stump gave out a very weak constrictor effect, while the dilator fibers were still nearly as effective as in normal animals. After 56 days (no tests were apparently made by these authors between 14 and 56 days) no constriction whatsoever could be obtained, nor did pilocarpin give any contraction of the denervated side of the lung.

The same fact is indicated in the second and third series of experiments, but in those experiments the vagus was resected after the animals had been sensitized for thirteen days and fifty-five days respectively. Now strictly interpreted, these two series show only that the vagus degeneration does not prevent a sensitized guinea pig's lungs from responding properly to the toxic injection. These two series, however, while not definitely proving the same point which the fourth series established, show other points of some interest.

In the second series of experiments, for example, the vagus was resected thirteen days after sensitization. The toxic dose was injected thirty to fifty-seven days after vagus section. As already stated, the normal response of an anaphylactic guinea pig was obtained. It may, therefore, be concluded that degeneration of the vagus nerve, after sensitization has about reached the lower limit of effectiveness,[18] exerts no marked effect on the response to the toxic injection

In the third series of experiments, one vagus was resected fifty-five days after the sensitizing dose was injected, and the toxic dose was given intravenously six to fourteen days after the vagus section. The animals behaved normally, that is, they died in the same fashion as normal, sensitized guinea pigs. But the toxic dose was injected at a time when vagus degeneration was far from complete, and in the first three animals at a time when the bronchodilator fibers were still physiologically active, while the activity of the broncho-constrictor fibers was also surely much impaired.[19] If the injected toxic dose, therefore, affected the nerve-endings it might be argued that the conditions were favorable for a bronchial dilatation. This did not occur; the three animals injected after six days of vagus degeneration showed typical anaphylactic lungs after death. In addition one of these animals had received a prophylactic subcutaneous injection of four milligrams of atropin sulphate, yet this animal showed only some delay in the onset of death, and its lungs

[18] As stated before, 14 days is practically the smallest time interval between sensitizing and toxic doses, the latter intravenous, which must elapse before the typical chain of symptoms ending in death may be obtained with some certainty.

[19] Dixon and Brodie, *loc. cit.*, 141.

revealed no evidence of any collapse. This hypothetical bronchodilator effect, due to a stimulation of the nerve endings, receives no experimental support from these data.

In all the experiments reported, there are no definite data which point to a sensitization of the vagus motor endings in the lung. This, of course, does not mean that such a sensitization may not occur. The solution of this section of the problem is unfortunately complicated by the fact that muscular sensitization does occur. Now in order to judge of an effect on the nerve endings, we must rely upon the response of the indicator-organ of the nerve endings, the muscle fibers, for it is impossible to observe the nerve endings alone. But if the indicator organ itself responds, independently of the nerve endings, it is readily seen that no answer will be obtained regarding an action on the endings. This is the case with horse serum sensitization in guinea pigs.

SUMMARY.

Depriving the bronchial muscles of one side of the lungs of their motor innervation does not interfere with sensitization nor the production of the typical anaphylactic lung. There is, therefore, direct sensitization of the muscle substance.

Complete degeneration of the vagus nerve, *after* sensitization has occurred, interferes in no appreciable way with the course of immediate anaphylaxis.

Partial degeneration of the vagus nerve, so that the bronchodilator fibers are still physiologically active, *after* sensitization has taken place, exerts no effect on the symptoms and lung picture of immediate anaphylaxis (very acute anaphylaxis with death after some minutes).

No definite evidence was obtained regarding the function of the motor nerve endings in the bronchial muscles in anaphylaxis.

The two sides of the lungs in immediate anaphylaxis may occasionally be unequal; when this occurs, the left side, especially the lower lobe, is usually fuller than the right side.

EXPLANATION OF PLATES.

PLATE LV.

FIG. 1. Typical anaphylactic lungs in a guinea pig. Magnification about 2×. The animal which furnished this lung was sensitized Nov. 17, 1909, by the subcutaneous injection of one cubic centimeter of horse serum. The right vagus was resected Jan. 11, 1910. The toxic dose (0.3 cubic centimeter 10 per cent. heated horse serum) was injected into the jugular vein Jan. 24.

It will be noted that the two lung sides appear about equally full. This lung is indistinguishable from normal anaphylactic lungs.

FIG. 2. Both lower lobes of a sensitized pithed guinea pig were placed each in an oncometer, artificial respiration being maintained. The heart stopped beating about five minutes before this tracing was obtained. The expansion and collapse of the lobes were registered by Marey tambours; upstroke = inflation. The upper curve was obtained from the lower right lobe and the lower curve from the lower left lobe of the lungs. Both vagi were cut.

During the time marked by broad, white bands, the peripheral stumps of either the left or right vagus were stimulated. Time = 6 second intervals; l. p. v. = left peripheral vagus; r. p. v. = right peripheral vagus. Petzoldt coil with two Daniell cells.

PLATE LVI.

FIG. 3. Same animal as in Fig. 2, but the animal had been dead about thirty minutes when this tracing was obtained. Interrupted stimulation of the left and right peripheral vagus stumps with the secondary coil pushed home still yielded definite broncho-constrictor effects, localized to that side of the lung on which the vagus was stimulated. Time = 6 second interval. Note long after-effect.

FIG. 1.

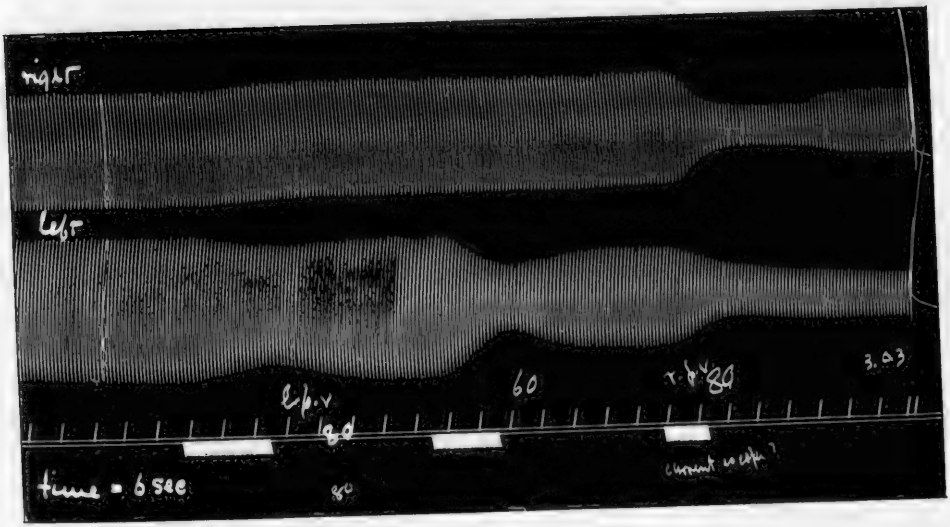

FIG. 2.

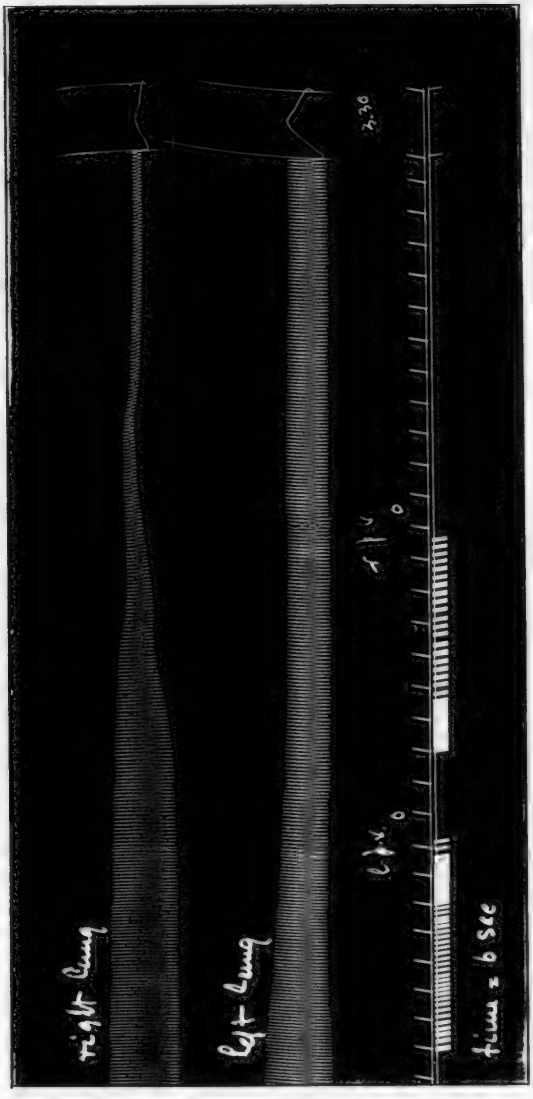

FIG. 3.

THE CULTIVATION OF THE LEPROSY BACILLUS AND THE EXPERIMENTAL PRODUCTION OF LEPROSY IN THE JAPANESE DANCING MOUSE.[1]

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PLATES LVII–LX.

Many attempts have been made to grow upon an artificial medium the acid-fast bacilli that are associated with the lesions of human leprosy, and not a few observers report success. However, confirmation is lacking and it may be said that multiplication of the leprosy bacillus *in vitro* up to the past year has failed. Occasionally an increase in the number of bacilli has been noted, at least for a period, in removed bits of the infected leprous tissue which have been transferred to an artificial medium, but growth in sub-cultures from these has invariably resulted negatively. The transference of parts of tissue along with the bacilli in all likelihood explains many of the recorded successes with the artificial cultivation of the leprosy bacillus.

The most important work on the cultivation of *B. lepræ* is by Clegg[2] who announced his success in the early part of last year. This author claims to have grown an acid-fast bacillus from the human leprous tissues with ameba and their symbiotic bacteria. Not only did he succeed in obtaining an initial growth but he was able to keep the bacilli multiplying in sub-cultures.

Of equal importance is the recent work of Sugai,[3] who, in a series of experiments with the Japanese dancing mouse, demonstrated that the leprosy bacilli not only multiply at the site of inoculation but become disseminated throughout the animal's body, pro-

[1] Read before the American Society of Pathologists and Bacteriologists, Washington, May 4, 1910. Received for publication June 1, 1910.

[2] *Philippine Jour. of Science*, 1909, iv, 403.

[3] *Lepra*, 1909, viii, 203.

ducing histologically the typical leprous lesion. The successful transmissibility of leprosy to this species of mouse affords an excellent opportunity to determine whether or not the acid-fast organisms cultivated upon an artificial medium are in reality leprosy bacilli.

In October, 1909, I began some experiments upon the artificial cultivation of the leprosy bacilli from human tissue. My object at first was to confirm if possible the work of others, and to attempt further to grow the bacilli directly from the tissues without the aid of symbiotic organisms, and to prove by animal inoculation the identity of the culture.

The leprous material was obtained from cases at the State Leper Home, located about sixty miles from New Orleans, at which institution every facility was afforded me in carrying out the experiments. There are about seventy-five inmates at the Home, among whom are found all types and stages of the disease. Sister Benedicta and her staff, who are in charge of the Leper Home, did much to facilitate the work, and I take pleasure in expressing to them my appreciation of their interest and coöperation.

The leprous tissue for the cultivation experiments was selected from four different cases: namely, (1) leprous nodule from the dorsal surface of the hand in a well-advanced case of tuberculous leprosy; (2) pus from a subcutaneous leprous abscess of the forearm in a typical case of leprous fever; (3) the organs (spleen, liver and ear) removed at autopsy from a fatal case of leprosy; and (4) leprosy nodules from the forearm of an early case of leprosy. In each instance every care was observed to remove the material aseptically; the skin surface was prepared in the usual manner as for surgical operation and the tissue excised with sterile instruments.

In each case the excision of the "nodules" gave rise to little or no pain, owing to the extreme anesthetic condition of the skin. The removed masses were then seared in the free flame to destroy any possible surface contaminating bacteria, after which they were incised and the central part carefully macerated with a sharp sterile curette, and the tissue juice suspended in sterile normal salt solution. From these suspensions the various culture media were inoculated.

The smear preparations stained in carbol-fuchsin and treated with Gabbot's solution always showed innumerable acid-fast bacilli arranged for the most part in small clumps and corresponding in morphology to *B. leprœ*. It is noteworthy that the clumps of bacilli even after prolonged maceration of the tissues in a mortar remained more or less intact. In the sections which were fixed in Zenker's fluid and stained after the methods used for the detection of acid-fast bacilli in the tissues, the organisms, with few exceptions, occurred in definite clusters and within the cells. A further microscopic study revealed these bacillary clumps almost invariably associated with cells, whose size and preservation seemed to depend upon the number of their enclosed bacilli, which bears out the opinion held by Hansen that leprosy bacilli are originally ensconsed in the cells and only appear in the tissue spaces when the normal relations are disturbed. Some of the cells contained only a dozen or fewer rods, and in these the outline of the cell and its nucleus was readily discernible. Usually, however, the bacillary masses were larger, consisting of twenty or more bacilli, arranged in a curious manner within the cell, and more often than otherwise within the nucleus. The characteristic grouping of the bacilli in bundles like packets of cigarettes was observed, but the usual arrangement was in the form of concentric rows. Even in the cells that contained the smaller clumps, this concentric massing of the rods was remarkable. It may be mentioned in this connection that as the leprosy bacilli occur so rarely outside the host-cell it is reasonable to believe that the latter furnish the essential nutriment for the bacilli, perhaps a nuclear ferment or globulin. This special affinity of the bacilli for certain cells led me to prepare an artificial medium composed in part at least of substances which are the result of cell metabolism and the end products of proteid digestion.

Whether the leprosy bacilli invade the cells and, through purely mechanical irritation or stimulation, the result of toxin, cause in turn division, which owing to the abnormal condition thus brought about is imperfect in character, giving rise merely to repeated division of the nucleus without division of the protoplasm, or whether the cells are in nature true phagocytes that have engulfed the bacilli, is a question still unsettled and one worthy of

further study. The former conception seems more likely to be the explanation, as all sizes and stages of preservation of these acid-fast bacilli-carrying cells can be followed in the early leprous lesion. That the bacilli continue to multiply in the cells, there can be no question and this is further proof in support of such a contention. Experimental work along this line is already in progress with the Japanese dancing mouse, which animal offers exceptional material to study the early cell changes.

The emulsified leprous material used for the inoculation of culture media contained few if any scattered bacilli; almost invariably they were transplanted in small clumps because of the inability to break them up, a feature which, as I shall presently point out, greatly aided the subsequent study to determine whether or not the transplanted leprosy bacilli are those that have actually multiplied.

The material employed in another series of experiments was obtained from a subcutaneous abscess in a case of so-called "leprosy fever." A word here on this case is of interest, as it illustrates that the leprosy bacillus may occasionally play a pyogenic rôle. The patient at the time had a temperature ranging from 102°–104° F., due apparently to the absorption of toxin from the large fluctuating tumors that occurred under the skin in various parts of the body. These subcutaneous abscesses were situated chiefly on the arms and face, and on opening discharged a thick yellowish white material in every way identical to ordinary pyogenic pus. On microscopic examination the pus consisted almost entirely of polymorphonuclear leucocytes together with small clusters of intra-cellular acid-fast bacilli (the so-called globi). The condition of the patient at the time was regarded as a secondary pyogenic infection, but subsequent bacteriological examination did not bear out the diagnosis. Repeated search through the smears prepared and stained in a variety of ways failed to show any bacteria other than the small "beaded" acid-proof rods which were present in innumerable masses. Besides, the material transplanted upon a variety of media remained negative with respect to growth after three or four weeks incubation.

At autopsy, tissue cultivation of *B. leprœ* was attempted from the scrapings of the excised lobe of the ear and from macerated

portions of the spleen and liver. Though separate sets of cultures were prepared from each, growth of an acid-fast bacillus resulted only from the ear nodules. Here the bacilli were extremely numerous as compared to the number in the spleen and liver, which might, in a way, account for the failure to obtain growth from the latter. As Clegg points out, and it has also been my experience, by far the greater number of leprosy bacilli transplanted fail to multiply (a not infrequent occurrence with many of our better known bacteria); this observation shows the necessity of carrying over in subculturing large numbers in order to insure growth.

A great variety of media was employed in the initial attempt to cultivate the leprosy bacillus from the human tissue, and duplicate series of each were placed under aërobic and anaërobic conditions at temperatures 37° C., 32° C. and 20° C. respectively. Suffice it to mention in detail only the media upon which multiplication of the bacilli resulted.

The sloped surface of plain water agar 1 per cent. alkaline to phenolthalin was first seeded with a mixture of entameba coli and *B. typhosus* and kept at room temperature for forty-eight hours to allow the ameba to increase in number. The tubes were then inoculated with several loopfuls of the emulsion prepared from the leprous tissues. Similarly, agar slopes were prepared after the method of Musgrave and Clegg for the cultivation of ameba, and later they were seeded with a portion of the suspension of leprosy bacilli; also separate tubes of nutrient agar to which was added 1 per cent. human defibrinated blood and seeded with a pure growth of *B. influenza* and the meningococcus were used. Again cultures of *B. coli, typhosus* and *dysenteriæ* and other intestinal bacteria were employed separately and in association upon a variety of media. Multiplication of an acid-fast bacillus occurred in all the mixtures that contained ameba and also upon the media seeded with influenza bacilli and meningococci.

With regard to the ameba cultures it did not seem to matter what nutrient was used, as growth resulted in every instance and attained macroscopic colony-form after a longer or shorter period; nor did the reaction of the medium appear to make any material difference. The temperature, however, was important, for only the cultures

kept at 32° to 35° C. gave rise to growth that could be distinguished by the naked eye. With certain of the successful initial cultures it was a question, in my mind, whether the media was in any way responsible. The fact that the bacilli increased in number upon various media inclined one at first to attribute the result to the bits of leprous tissue unavoidably carried over with the bacilli. Certainly this accounted for growth on the media seeded with influenza bacilli and meningococci, as afterwards verified by the failure to get subplants to multiply.

It was noteworthy with the ameba cultures that growth of the bacilli continued for months without reseeding with ameba, which would seem to argue that multiplication did not depend solely upon the active ameba or upon any substance elaborated by them, for under the circumstances it is natural to suppose that even where an abundance of by-product is present, it would be used up sooner or later and the bacilli would of necessity cease further to multiply.

Cultivation was next attempted with ameba alone, which was accomplished by treating a heavy suspension of ameba that had been grown in conjunction with the typhoid bacillus with a strong bacteriolytic typhoid serum whereby the typhoid bacilli were completely killed off, as was subsequently shown by the control tests. Sloped agar was then heavily coated with pure ameba and inoculated with leprosy bacilli. By this procedure it was possible to determine whether the growth of *B. leprœ* depended upon the active or inactive ameba. It had been previously determined that the leprosy bacilli would not multiply in the presence alone of typhoid organisms or their autolized product, but that they would multiply in a mixture of ameba and typhoid bacilli and continue to do so for months after the ameba had become encysted; this observation showed conclusively that the protozoa alone were responsible for the growth in this particular instance, and that the encysted forms answered the purpose equally as well as the active vegetative types.

Having determined that the leprosy bacillus would live and continue to multiply indefinitely outside the animal body in the presence of ameba alone, it was next attempted to cultivate them upon a medium without living organisms. Since the leprosy bacilli appear to live in commensal relation with the host entering the tissue cells

and feeding upon substances utilized by them or upon products of cell metabolism, it was thought that a medium simulating the conditions enjoyed by the bacillus under its natural environment would result favorably in artificial growth, provided the proper temperature and moisture were maintained. Based on this hypothesis, a medium was prepared and compounded of substances such as tryptophane, cystein, leucein, etc., with the view of supplying an artificial food analogous to that met with in the tissues. It was further thought that if these substances in varying combinations could be used in the presence of some medium where excessive oxidation takes place, that the condition *in vitro* would be all the more enhanced. Consequently the banana was selected for the purpose.

The medium was prepared as follows: The rind was carefully removed from the fruit portion of fully matured green bananas, every precaution to avoid contamination being used, and large blocks of the fruit, after slanting one surface with a sharp knife, were introduced into suitable sterile glass cylinders provided at the bottom with cotton plugs saturated in sterile distilled water. These plugs served not only as support for the banana but as a source of constant supply of moisture. Sterile 1 per cent. solutions[4] of tryptophane, cystein (made from protein) and leucein were next prepared and a portion of each poured upon and allowed to saturate the banana. These solutions were used separately and in varying combinations, in order to determine upon which the *B. leprœ* would grow best or grow at all. Also ten cubic centimeters of a 2 per cent. nutrient agar of a titer 1.5 per cent. alkaline to phenolphthalin was melted down and five cubic centimeters of a 2 per cent. solution of the above mentioned substances were added, thoroughly mixed and solidified in the slanted position.

Both the banana and agar, which was saturated in a 1 per cent. solution of cystein, proved an excellent medium for the artificial cultivation of the leprosy bacilli when incubated at 32° to 35° C. The maximum growth occurred at a temperature of 32° C. Light seems to favor the growth of *B. leprœ;* cultures kept in a glass incubator regulated at 32° C. grew more rapidly than those in the

[4] To procure these substances bacteria-free, the solutions are passed through a Berkefeld filter; heating apparently destroys their nutritive value.

dark chamber under similar conditions. Multiplication began early in the transplants and visible growth developed in the form of small glistening white colonies in from four to six weeks. Growth also occurred upon the banana and agar where a solution of cystein and tryptophane had been added. The fact that growth occurred on the protein-cystein medium, and not upon the others except in the presence of it, shows very conclusively that *B. leprœ* utilize the end products of digestion and not the products of cell metabolism. At least it is reasonable to assume that this is the case, if deductions may be drawn from these experiments.

Multiplication *in vitro* of an acid-fast organism was obtained from the transplanted leprosy tissue on the above mentioned media from four cases of leprosy which corresponded in every essential to the leprosy bacillus. Not only did the leprosy bacilli develop in the original cultures but they continued to grow in subcultures. That the artificial growth is *B. leprœ* there can be no doubt, as the morphological and cultural features and the animal tests have clearly proved.

CULTURAL FEATURES.

Colonization of the leprosy bacillus occurs on the surface of special medium after four to six weeks incubation at 32° C. in the form of moist semi-translucent greyish white colonies; prior to this period growth can only be detected on microscopic examination. The colonies are distinctive in their macroscopic appearance and unlike those of any known acid-fast organism. At first they appear to the naked eye as glistening white specks and these slowly increase in extent until they attain a size two millimeters in diameter. The colonies are often heaped up in the center, which causes them to be elevated above the surface of the medium; at no period are they flattened, though the periphery thins out imperceptibly. They show, however, an irregular wavy margin on microscopic examination. The colonies are not easily broken up; on the contrary, it is with greatest difficulty that they are divided, though they can be readily removed from the surface of the medium *en masse*. Even when growing with ameba and their symbiotics, they are easily distinguished from the associated colonies of other bacteria, owing to their glistening greyish white appearance.

With the initial culture obtained from the leprous nodule where innumerable microscopic bacillary masses were transferred, only a few developed into colonies that could be recognized by the naked eye, and these occurred sparsely distributed over the surface of the medium, and not all of them continued to full development. It is noteworthy that even on microscopic examination of stained smears prepared from parts of the medium where there was no apparent visible growth many of the transplanted globi had increased in size. Instead of the original bacillary clumps of a dozen or more rods they now presented larger masses, irregular and wavy in outline, and composed of innumerable bacilli (Plate LVIII, Fig. 4). In the majority of the cultures, the concentric arrangement of the bacilli, so common in the tissues, had entirely disappeared, the organisms now assuming thick mattress-like collections so dense at the centers that the individual bacilli could not be differentiated. There are still to be seen, however, in the microscopic field, clusters of bacilli of the original size and concentric arrangement, which are apparently dead and give no evidence that multiplication had begun (Plate LVIII, Fig. 3). The unaltered appearance of these clusters made the recognition of growth in others all the more easy, as they served for comparison. In none of the cultures was there visible growth before the fourth week, although an increase in number of bacilli was noted on microscopic examination in twelve to fifteen days after inoculation, as determined by the increase in size of the globi, and change in morphology of the newly formed bacilli.

It is surprising on what a variety of media the leprosy bacilli in the initial plants continue to multiply, especially in the presence of light and where a moist temperature is maintained. As already mentioned, this is accounted for in the fact that the bacilli find food in the tissues that are unavoidably carried over in the transference. All the successful cultivations occurred in a moist temperature ranging between 32° and 35° C. In my experience the temperature and light is most important, more so perhaps than any other factor for the success in the cultivation of *B. leprœ* outside the animal body. The organism at best grows slowly, much more so than the tubercle bacillus. It is essential to keep the medium

from drying out during the long period of growth, and this is accomplished by placing the inoculated medium in a recumbent position in a moist chamber. The culture should not be sealed in any way, as oxygen is constantly required.

A series of duplicate cultures in each lot of experiments were simultaneously placed at 37°, 32° and 25° C., respectively, and observations made at regular intervals to note whether any change in number and character of the bacilli had occurred. In no case was I able to detect, even on microscopic examination, that the bacilli in the cultures at 37° C. had increased, no matter what medium was employed; the transferred clumps remaining approximately of the same size and character for weeks. It was noted, however, in the cultures where the transplanted bacilli did not grow, that many of them after several weeks slowly underwent a form of granular metamorphosis, though the chromatin masses still held with equal intensity their acid-resisting property, but all semblage of bacillary forms had disappeared. The globi now appeared as spherical masses of small red staining granules of varying sizes and somewhat resembled cocci. Even in the cultures where the bacilli had developed into microscopic colonies, when placed at 37° C. further growth would cease and these in the course of a few weeks would lose their glistening appearance and become converted into dry brownish red masses. A comparative microscopic study of the cultures from week to week served admirably to clear up any doubt as to their origin. In this way it was readily determined by the increase in size of the clumps and the morphological differences between the old and new bacilli that the growth originated in the globi.

The question naturally arises in the work on leprosy cultivation as to the identity of the acid-fast bacilli that develop upon an artificial medium. Are they not contaminating organisms introduced at the time of inoculation or already present in the medium? Had the transplanted bacilli been single and diffusely scattered over the medium, it might well lead to a mistake in the origin of the resulting artificial growth, especially if there had been other bacilli of the acid-proof variety associated, but the fact that in all my experiments the transplanted bacilli were in discrete colony masses,

whose gradual increase in size could be followed day by day until macroscopic colonies resulted, would hardly permit of any other interpretation. Again, there can be no question that the artificial cultures are tubercle bacilli, which was clearly proven by the following control tests. Guinea pigs and rabbits inoculated subcutaneously and intraperitoneally with quantities of the leprous tissue failed to develop or show microscopically any evidence of tuberculosis or other lesions; besides, the cultivated bacilli, when introduced into these animals, did not grow or produce lesions. In addition, a variety of favorable media, including Dorset's egg mixture, was used for the isolation of the tubercle bacillus and in each instance remained negative, though the material was especially suited for the direct recovery of the tubercle bacilli from infected tissue, in that there was an absence of pyogenic or other bacteria, as shown by microscopic examination and by various cultural tests. Air contamination is hardly probable and can, I think, be excluded. The fact that the acid-fast bacillus recovered from four different cases was identical would render it extremely improbable that it was an extraneous contamination.

Morphology.—As a rule, the size, shape and character of the artificially grown leprosy bacillus corresponds fairly well to those in the human tissues. There are differences, but they are too inconstant to permit of the contention that *B. lepræ* cultures completely change in morphological features. In general, it may be stated that the cultivated bacilli are longer, more curved and show a greater irregularity in the distribution of the chromatin than those in the tissues, where they are short, slender and slightly curved (Plate LVII, Figs. 1, 2). Again, in the tissues of the four cases studied, the individual organisms usually occurred in parallel rows ensconsed in cells, each row consisting of two or more bacilli arranged one behind the other. Often the chromatin was more compact at the extremities, and consequently there is a lighter constricted appearance of the central segment. This bipolar condensation of the chromatin was all the more accentuated on lengthening the time of decolorization, whereas each row of two or four bacilli now appeared to be twice that number. Even with single isolated organisms, it was surprising that what ap-

peared to be solid rods were rendered segmented by prolonging the decolorization. Other bacilli, and by far the fewer, showed a distinct tapering of one or both extremities and a denser massing of the chromatin in a ragged manner along the central portion, giving rise to the occurrence of barred spindles with pointed ends. The bacilli show no distinct granules, but what appear to be granules are in reality denser collections of the chromatin. In the tissues the individual leprosy bacilli are not distinguishable morphologically from the tubercle bacilli, unless perhaps they are smaller and more slender. The enormous number in the cutaneous nodules and their characteristic arrangement within cells is unusual for tubercle bacilli to say the least, and especially in the skin lesions of tuberculosis.

The artificial cultures of leprosy usually present a delicate filamentous arrangement of the bacilli, especially where they have become accustomed to a saprophytic existence; bacilli are slightly curved and often contain distinct metachromatic granules analogous to those met with in certain forms of the diphtheria bacillus (Plate LVIII, Figs. 6 and 7; Plate LIX, Figs. 8, 9, 10). The rods may be twice or three times the length of those in the tissues, although they do not differ greatly in thickness. Often in the same field all variations in size and shape of individual bacilli are met with. Commonly they describe a slender curve with tapering extremities. All forms occur from solidly stained coccoid shapes to slender and slightly curved filaments with numerous chromatic segments and, occasionally, metachromatic granules. The morphological transformation on the whole is striking, but no more so than what occurs in other of our better known bacteria, for example, the diphtheria and tubercle bacillus. The slender metachromatic filaments may be regarded as the most common morphological character of *B. leprœ* under artificial cultivation; at least this was the form most commonly met with in my cultures. Not all the bacilli are filiform or contain granules; many are short with pointed ends, and the chromatin is equally distributed throughout the body of the organism. This change in morphology I take for pleomorphism and I regard it as a natural sequence of altered environment, and analogous to the bipolar and transverse massing of the chromatin in

certain types of the Klebs-Loeffler's bacillus. These forms are also similar in many respects to some strains of tubercle cultures. Other bacilli grow in slender curved filaments composed of three or more chromatin segments arranged with respect to one another in closely parallel columns (Plate LIX, Fig. 11). Branching at no time was detected in the cultures. Clear spaces occur in the bacilli, but they are not to be regarded as spores. Such spaces are the result entirely of the massing of the chromatin. The great diversity of form assumed by the leprosy bacillus when grown under artificial conditions would argue that it is truly pleomorphic and to be classed among the higher bacteria.

Tinctorial Properties.—The staining reaction of *B. leprœ* from culture is very much like that of the tubercle bacillus. It takes the stain more readily than the latter and decolorizes more quickly, but the difference is not sufficient as a means of differentiation. Some observers who claim to have cultivated *B. leprœ* state that in cultures the bacilli lose the power to retain the stain after the ordinary treatment with weak acids, etc.; in other words, the artificially grown organism is no longer acid-fast. I have never been able to detect the slightest difference in respect to "acid-fastness" between the bacilli leading a saprophytic life and those in the human tissues. The stained bacilli from cultures are, if anything, more resistant to decolorizing fluids, owing to an apparently greater amount of fatty deposit or capsule about them. My cultures on artificial medium which are now in the twelfth generation and more than seven months out of the animal body show no change whatever in respect to their ability to retain the stain when treated with Gabbot's solution or when treated with any of the more common decolorizing agents. Undoubtedly the non-acid proof cultures that have been described by some observers are extraneous microörganisms and not *B. leprœ*. They do not, however, resist to the same extent the acid treatment, as in the case of tubercle cultures, any more than they do in the tissues. Thirty per cent. nitric acid followed by 95 per cent. alcohol will completely decolorize the stained leprosy bacilli in the same length of time that tubercle bacilli resist this treatment, but in the case of the ordinary bath in Gabbot's solution for two minutes, after thorough staining in carbol-fuchsin, they are

not decolorized; and they resist the treatment with weak acids and alcohol alone for an indefinite period. Like the better known organisms of the acid-resisting group, the leprosy cultures are positive to Gram's method of staining. The chromatin masses in the individual rods retain more intensely the methyl violet stain; with the ordinary simple dyes they exhibit less chromatin segmentation and appear more solidly stained throughout.

ANIMAL EXPERIMENTS.

A variety of animals was employed to determine the pathogenicity and identity of the cultures isolated from these cases of human leprosy, including guinea pigs, rabbits, mice and rats. In each case separate animals were inoculated intra-peritoneally and subcutaneously with heavy emulsions of bacilli prepared from the leprous tissue and from cultures. In no instance where the culture or infected tissue had been used for the test was there any appreciable effect following the inoculation that could be attributed to the organism in question, with the exception of the Japanese dancing mice. Although many of the animals were kept under observation for months, no acid-fast bacilli nor microscopic tissue changes were noted at autopsy either at the site of inoculation or elsewhere in the body.

On the other hand, the dancing mice develop typical leprous lesions in four to eight weeks after subcutaneous or intra-peritoneal injection with *B. leprœ*. Not only were the results positive in the animals where the inoculation was made with emulsions from the human leprous tissue, but also with the artificial cultures. So far eight of these mice, seven males and one female, have been used in the experiments and all have developed leprosy. The dosage consisted of .5 cubic centimeter of an emulsion of the leprous tissue or, in the case of cultures, one colony (one to two millimeters) thoroughly emulsified in .5 cubic centimeter of sterile salt solution. The mice were killed at intervals of four, six, eight and ten weeks. It is noteworthy, however, that during this period none of the animals showed the slightest evidence of illness; at all times eating and dancing about the cages as usual.

In my experience so far with subcultures of *B. leprœ,* I have

not been able to note any difference in degree of infectibility. Cultures many generations removed from the parent stem, and more than six months under artificial cultivation, give rise to lesions in the Japanese mouse about as quickly as the bacilli of the primary culture or those of the human tissue.

The earliest macroscopic change was observed in mice which had been inoculated only four weeks, and in those receiving an intraperitoneal injection. Here the lesions appear as discrete, glistening, white nodules one to three millimeters in size and firmly attached to the serous surface of the peritoneal organs (Plate LX, Fig. 13). At this period of the infection there are no demonstrable lesions in the viscera, though later (eight to ten weeks) they occur disseminated throughout the body, the spleen and lymph nodes more especially showing the greatest involvement (Plate LX, Figs. 12, 15, 16).

The histopathology of the experimental lesions is identical in its appearance to human leprosy. At first the lesions consist in discrete foci of lymphoid and plasma cells, the latter predominating (Plate LX, Fig. 14). Sections from the older nodules are composed chiefly of epithelioid cells, fibroblasts and multinucleated elements, the so-called "lepra-cell" (Plate LX, Fig. 12). Acid-fast bacilli are present in the sections in enormous numbers and occur for the most part within cells. Even in the earliest change, which consists in lymphoid and plasma cell collections, these cells are filled with bacilli (Plate LVII, Fig. 1). Caseation or necrosis does not occur, at least in the early nodules. A full description of the experimental lesions will appear in a later communication.

SUMMARY AND CONCLUSIONS.

Pure cultures of an acid-fast bacillus were cultivated upon special media from the human tissues in four cases of leprosy. The nature of the growth, morphological characters and tinctorial properties do not differ for any of the cultures and correspond closely to the bacilli in the human leprous tubercles.

That the bacillus of leprosy will multiply and continue to do so indefinitely outside of the animal body was first demonstrated by Clegg who cultivated an acid-fast organism from leprosy tissue in

the presence of ameba and their symbiotics. Not only have I been able to confirm Clegg's work, but in addition I have succeeded in growing the bacillus in pure culture and in reproducing the disease in the Japanese dancing mouse, thereby establishing its identity. This species of animal acquires the infection in four to six weeks after intraperitoneal or subcutaneous inoculation with either emulsions of fresh leprous tissue or the pure cultures of *B. leprœ*. Comparatively few bacilli are necessary to infect the mouse; and the mode of inoculation does not seem to make any appreciable difference in respect to the nature and time of development of the lesion.

The experimental lesions are proliferative in character and identical with those in the human subject. Macroscopically they appear as glistening, white nodules which, in the early stages of development, resemble miliary tubercles.

In my experience neither the cultures nor the bacilli directly from the human tissues have shown any evidence of multiplication or given rise to lesions when injected into the ordinary laboratory animals such as guinea pigs, rabbits, gray and white mice and rats, although repeated attempts have been made to infect these animals.

B. leprœ will not only multiply but it will colonize on a plain agar medium seeded with a pure culture of encysted ameba (Plate LVIII, Fig. 5), and upon an agar or banana medium prepared with a 1 per cent. solution of cystein and tryptophane. Colonization occurs in the form of glistening, white colonies, one to two millimeters in diameter, in from one to two months incubation.

The bacilli in cultures are at all times acid-fast and differ only in morphology from those of the tissues in that they exhibit a greater variation in the distribution of the chromatin and are longer and more distinctly curved.

To prove that the cultures obtained from the human tissues of these four cases are leprosy bacilli and not some other acid-fast species, the following facts are offered: (1) the growth features are distinctive and multiplication takes place only under special conditions of temperature and medium; (2) the complete correspondence in tinctorial properties and similarity in morphology to those in the tissues; (3) the failure to multiply or produce lesions in the common laboratory animals; and (4) the growth of the bacilli and

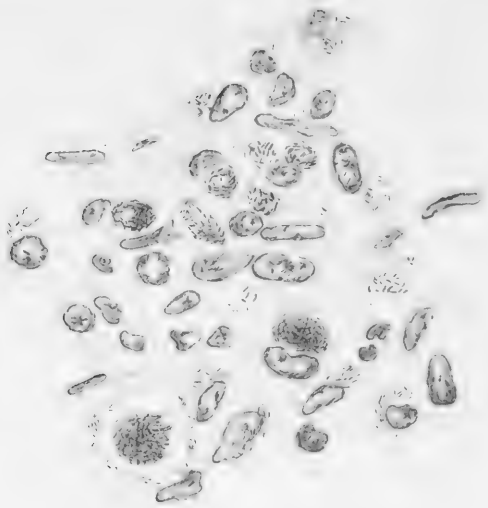

FIG. 1.

FIG. 2.

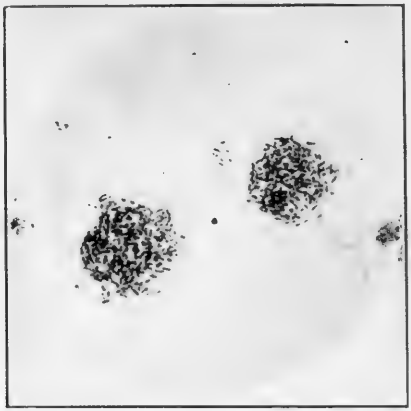

FIG. 3.

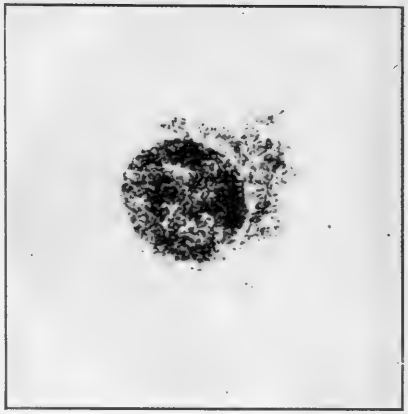

FIG. 4.

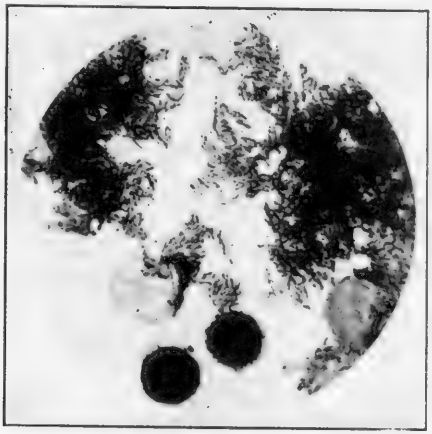

FIG. 5.

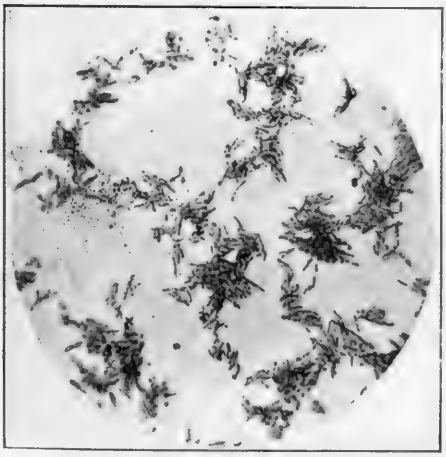

FIG. 6.

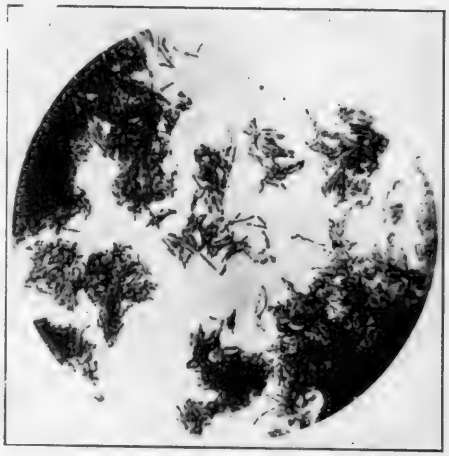

FIG. 7.

FIG. 8.

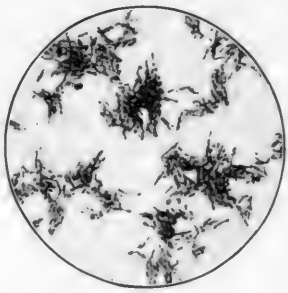

FIG. 9.

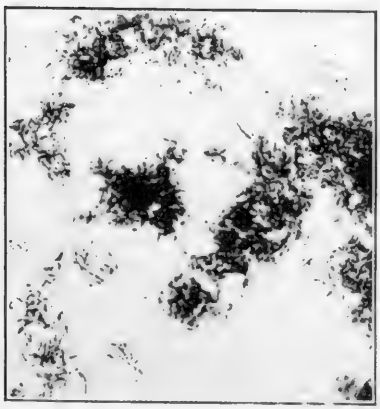

FIG. 10.

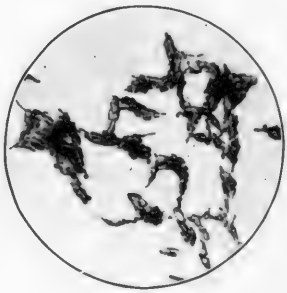

FIG. 11.

the production of typical leprous lesions in the Japanese dancing mouse.

The successful cultivation of *B. lepræ* and the fact that the cultures retain pathogenic properties are of commanding importance in respect to a possible production of an artificial immune serum for combating the infection in man. Work along this interesting line is already in progress in our laboratories.

EXPLANATION OF PLATES.

PLATE LVII.

FIG. 1. Colored drawing from microscopic section of experimental leprosy in the omentum of the Japanese dancing mouse.

FIG. 2. Smear preparation from pure culture of *B. lepræ* to illustrate the usual morphology and arrangement of bacilli under artificial cultivation.

PLATE LVIII.

FIG. 3. The development of isolated colonies from the so-called globi. Note also in the field two smaller bacillary clumps which apparently have failed to develop.

FIG. 4. Leprosy colony showing a further stage of development. Note the spread of bacilli from the periphery of the mass.

FIG. 5. Culture of *B. lepræ* growing with cysts of *Entameba coli.*

FIGS. 6, 7. Pure cultures of leprosy showing the characteristic morphology and arrangement of the bacilli.

PLATE LIX.

FIGS. 8, 9 AND 10. Pure cultures of leprosy showing the characteristic morphology and arrangement of the bacilli.

FIG. 11. Pure culture of *B. lepræ* presenting a curious ribbon-like arrangement in which the bacilli are end to end in wavy parallel columns.

PLATE LX.

FIG. 12. Experimental leprosy lesion in the mesentery of the Japanese dancing mouse. Note the large lepra cells and enclosed masses of bacilli.

FIG. 13. Leprosy tubercle on the surface of the spleen (Japanese dancing mouse).

FIG. 14. Early leprosy nodule in the mesentery (Japanese dancing mouse).

FIG. 15. Section from mesenteric lymph node of the Japanese dancing mouse showing early leprosy tubercule.

FIG. 16. Multiple leprous tubercles in the omentum of the Japanese dancing mouse.

INTRACELLULAR PROTEOLYTIC ENZYMES OF LIVER.[1]

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In all living tissues, protein constituents are continually being broken down within the body cells and protein decomposition products are excreted. If any or all of the processes of degradation occurring during life are the work of enzymes, we should expect to be able to demonstrate the existence of these enzymes in the tissues after death. The occurrence of such proteolytic enzymes was first established in 1890 when E. Salkowski[2] showed that liver, muscle and suprarenal glands, when minced and kept in chloroform water at 37°C. underwent what he termed "autodigestion." Somewhat similar observations had previously been made by Salomon[3] and by Schmiedeberg.[4] Jacobi[5] later confirmed Salkowski's observations and called the phenomenon "autolysis." These intracellular proteolytic enzymes are not known to be excreted into the body fluids but may be obtained in an active state in the press juice or in the filtrate of water extracts of organs.

Whether the phenomenon of autodigestion of organs removed from the animal body is related in nature to the physiological disintegration of protein within the living cell is a problem that is still unsolved. Parenteral digestion of protein has been observed. The rôle of leucocytes and their enzymes in effecting the removal of inflammatory products has been clearly pointed out by Opie[6] and others. The absorption of areas of necrosis not associated

[1] Presented before the Society for Experimental Biology and Medicine, April 20, 1910. Received for publication July 5, 1910.

[2] Salkowski, *Zeit. f. klin. Med.*, 1890, xvii, Suppl., 77.

[3] Salomon, *Arch. f. Anat. u. Physiol.*, 1881, 361.

[4] Schmiedeberg, *Arch. f. exper. Path. u. Pharm.*, 1881, xiv, 379.

[5] Jacobi, *Zeit. f. physiol. Chem.*, 1900, xxx, 149.

[6] Opie, *Jour. of Exper. Med.*, 1906, viii, 536.

with bacterial infection is probably largely accomplished by enzymes existing in the degenerating cells. Jacobi[7] has been able to demonstrate, in a lobe of liver deprived of its blood supply, the presence of leucin and tyrosin after thirty-six hours. In phosphorus poisoning and in acute yellow atrophy of the liver, protein decomposition products are found in large quantities in the degenerating organ. Such evidence of autodigestion has, however, been observed in pathological conditions in which cell nutrition, oxidation, and other chemical reactions have probably been profoundly disturbed so that an exact analogy to physiological processes cannot be drawn. These facts are nevertheless suggestive, and the possibility is apparent that these extremes of tissue degradation may represent but an intensified picture of normal protein metabolism. In fact, Lane-Claypon and Schryver[8] have been able to show that in hunger, where the animal's energy needs are supplied by his own tissue, autolytic digestion of liver is increased.

The influence of reaction upon autolysis of organs has been exhaustively studied. Investigation of this subject was first carried on by pupils of Salkowski shortly after the discovery of the process of autodigestion. Schwiening[9] has found that sodium carbonate inhibits the autolytic process, and Biondi[10] has demonstrated a favorable influence of hydrochloric acid on autodigestion of liver. Hedin and Rowland,[11] whose investigations were made with tissue plasma obtained by Buchner's method, have established that self-digestion in the majority of organs is facilitated by the presence of 0.25 per cent. acetic acid. It is depressed by the presence of alkalies, of calcium carbonate and of magnesium oxide. Wiener[12] has also demonstrated the sensitiveness of autolytic enzymes to alkali, and has shown that alkalinity corresponding to that of the blood practically checks their power to effect protein decomposition. From this observation he urges that proteolytic endo-enzymes can not be of great physiological importance during

[7] Jacobi, *loc. cit.*
[8] Lane-Claypon and Schryver, *Jour. of Physiol.*, 1904, xxxi, 169.
[9] Schwiening, *Virchows Arch.*, 1894, cxxxvi, 444.
[10] Biondi, *Virchows Arch.*, 1896, cxliv, 373.
[11] Hedin and Rowland, *Zeit. f. physiol. Chem.*, 1901, xxxii, 531.
[12] Wiener, *Cent. f. Physiol.*, 1905, xix, 349.

the normal life of the cell, and autodigestion of tissue in living organisms occurs only in areas in which the blood supply has been reduced. Such an opinion cannot be regarded as conclusive, since many investigators have observed the power of tissues to digest themselves in alkaline medium. In addition, proteolytic enzymes acting best in alkaline medium are well known in polymorphonuclear leucocytes, and Hedin[13] has been able to demonstrate the presence of such an enzyme in the cells of the spleen.

Baer and Loeb[14] have tested the effect of varying concentrations of acid and alkali upon autolysis of liver. Their experiments show the usual favoring influence of acid, and they have obtained a greater relative degree of digestion in alkali than has been observed by other investigators. They demonstrated an optimum of concentration of alkali in which the degree of autolysis is greater than that found in neutral medium. Because of this fact, they suggest the possible presence of an alkaline enzyme. Schryver[15] has found that the favorable influence of acid upon autolysis is dependent upon the absolute amount of acid added and is not specially affected by dilution. He thinks that acid effects a neutralization of tissue bases which control the activity of the autolytic enzyme. Hedin[16] has made the interesting observation that after pretreatment of certain tissues, namely, liver, kidney, thymus, spleen, muscle, and testicle, with 0.2 per cent. acetic acid and subsequent neutralization of the acid, autodigestion in 0.25 per cent. sodium carbonate is increased. Carrying these studies further with the spleen, Hedin has been able to demonstrate an anti-proteolytic action of expressed spleen juice. This anti-enzymotic action of the juice is exerted against the alkaline enzyme of the spleen, and is destroyed by pretreatment with acid, the increased digestion in alkali after acid treatment being thus explained.

Much interest is attached to the fact that, notwithstanding the presence of destructive agents in all living tissues, organs succeed in guarding their integrity. In recent years much attention has

[13] Hedin, *Jour. of Physiol.*, 1904, xxx, 155.
[14] Baer and Loeb, *Arch. f. exper. Path. u. Pharm.*, 1905, liii, 1.
[15] Schryver, *Biochem. Jour.*, 1906, i, 123.
[16] Hedin, Festschr. f. Olof Hammarsten, Upsala, 1906, vi, 1.

been centered on the anti-enzymotic properties of blood serum, and of organ extracts. Weinland[17] has shown that the stomach does not digest itself because of the presence of an anti-enzyme in the cells of the gastric mucosa. Intestinal worms resist the action of the digestive juices by means of the anti-enzymes which they contain. Hahn,[18] Landsteiner,[19] Hedin[20] and others have found substances in blood serum which are able to check the proteolytic action of trypsin. Opie[21] has demonstrated that a similar anti-enzymotic power over the alkaline enzyme of the polymorphonuclear leucocytes is possessed by the serum. Many recent studies have shown an increase in the anti-tryptic power of the blood in cachectic conditions, notably in cancer and in chronic pulmonary tuberculosis. The stimulus to the production of anti-tryptic substances has been variously assigned to enzyme freed from disintegrating leucocytes, to pancreatic trypsin and to increased intracellular enzymotic activity, or to combinations of the above factors. Blood serum has, likewise, an inhibiting effect upon the progress of tissue autolysis. The anti-tryptic action of the blood is, however, exerted only against alkaline enzymes such as trypsin and the enzyme of polymorphonuclear leucocytes. Hedin[22] has shown, on the other hand, that blood serum does not effect inhibition when added to that proteolytic enzyme of spleen which acts in acid medium. Since this enzyme is the more conspicuous of the autolytic enzymes the influence of blood serum upon autolysis appears somewhat limited.

Methods.—The tissues used in the following experiments were obtained from healthy normal dogs. Where normal tissue has not been used a special note is made. The animals were anesthetized with ether, and rapidly bled to death from the carotid arteries. The organ used for the study of autolysis was the liver. In certain instances beef serum denaturalized by heat was added to the autolysing mixture as substrate. The autolytic mixtures were in-

[17] Weinland, *Zeit. f. Biol.*, 1903, xliv, 45.
[18] Hahn, *Berliner klin. Woch.*, 1897, xxxiv, 499.
[19] Landsteiner, *Cent. f. Bakt.*, Abt. I, 1900, xxvii, 357.
[20] Hedin, *Jour. of Physiol.*, 1903, xxx, 195.
[21] Opie, *Jour. of Exper. Med.*, 1905, vii, 316.
[22] Hedin, *Jour. of Physiol.*, 1904, xxx, 155.

cubated in the thermostat at 37°C. for five days. Toluol was added in sufficient quantity to prevent bacterial growth. At the conclusion of the period of digestion the coagulable protein was precipitated by boiling with weak acetic acid and twenty per cent. magnesium sulphate. The coagulum was removed by filtering and the incoagulable nitrogen of the filtrate determined by the Kjeldahl method. The amount of digestion is expressed in cubic centimeters of N/10 sulphuric acid. Controls were made in each instance by boiling immediately equal quantities of the materials used and determining the incoagulable nitrogen of the filtrate. The figures given represent the amounts of digestion after the subtraction of controls.

AUTOLYSIS OF NORMAL LIVER IN MEDIA OF VARYING REACTION.

In a number of experiments the autolytic power of liver in acid, neutral and alkaline media was determined. The liver was minced immediately after the death of the animal and the power of self-digestion tested. In some instances denaturalized beef serum was used as substrate. All digestion mixtures were diluted to twenty-five cubic centimeters with 0.85 per cent. salt solution. A few of the experiments performed are cited as examples.

In Experiments I and II the animals were anesthetized with ether and bled to death from the carotid arteries. The liver was aseptically excised without perfusion and rapidly passed through a hashing machine. Beef serum denaturalized by heat was used as substrate. The digestion flasks were incubated for five days at 37°C.

EXPERIMENT I.

Quantity of Liver.	Medium.	Digestion.
2.5 gms.	0.2 per cent. acetic acid	36.6 c.c. N/10 H_2SO_4
2.5 gms.	neutral	6.7 c.c. N/10 H_2SO_4
2.5 gms.	0.2 per cent. sodium carbonate	3.5 c.c. N/10 H_2SO_4

EXPERIMENT II.

Quantity of Liver.	Medium.	Digestion.
2.5 gms.	0.2 per cent. acetic acid	29.4 c.c. N/10 H_2SO_4
2.5 gms.	neutral	7.9 c.c. N/10 H_2SO_4
2.5 gms.	0.2 per cent. sodium carbonate	2.0 c.c. N/10 H_2SO_4

In the above experiments the degree of digestion in 0.2 per cent. sodium carbonate is lower than that usually observed because of the inhibiting influence of the heated beef serum.

In Experiments III to VII no substrate was added. Otherwise, they resembled Experiments I and II.

EXPERIMENT III.

Quantity of Liver.	Medium.	Digestion.
2.5 gms.	0.2 per cent. acetic acid	20.7 c.c. N/10 H_2SO_4
2.5 gms.	0.2 per cent. sodium carbonate	3.6 c.c. N/10 H_2SO_4

EXPERIMENT IV.

Quantity of Liver.	Medium.	Digestion.
2.5 gms.	0.2 per cent. acetic acid	0.5 c.c. N/10 H_2SO_4
2.5 gms.	neutral	8.7 c.c. N/10 H_2SO_4
2.5 gms.	0.2 per cent. sodium carbonate	4.4 c.c. N/10 H_2SO_4

The total absence of digestion in acid medium in this experiment is difficult to explain, but is obviously referable to a technical error.

EXPERIMENT V.

Quantity of Liver.	Medium.	Digestion.
2.5 gms.	0.4 per cent. acetic acid	35.6 c.c. N/10 H_2SO_4
2.5 gms.	0.2 per cent. acetic acid	34.8 c.c. N/10 H_2SO_4
2.5 gms.	neutral	11.3 c.c. N/10 H_2SO_4
2.5 gms.	0.2 per cent. sodium carbonate	4.8 c.c. N/10 H_2SO_4
2.5 gms.	0.4 per cent. sodium carbonate	0.6 c.c. N/10 H_2SO_4

EXPERIMENT VI.

Quantity of Liver.	Medium.	Digestion.
2.5 gms.	0.2 per cent. acetic acid	23.7 c.c. N/10 H_2SO_4
2.5 gms.	neutral	9.2 c.c. N/10 H_2SO_4
2.5 gms.	0.2 per cent. sodium carbonate	12.2 c.c. N/10 H_2SO_4
2.5 gms.	0.4 per cent. sodium carbonate	10.4 c.c. N/10 H_2SO_4

The liver was carefully perfused with salt solution in the following experiment in order to determine the influence upon autolysis of the small amount of blood left in the vessels after bleeding. The duration of incubation was two days.

EXPERIMENT VII.

Quantity of Liver.	Medium.	Digestion.
5 gms.	0.2 per cent. acetic acid	43.1 c.c. N/10 H_2SO_4
5 gms.	neutral	11.9 c.c. N/10 H_2SO_4
5 gms.	0.2 per cent. sodium carbonate	7.4 c.c. N/10 H_2SO_4

The results obtained in the foregoing experiments are typical of autolysis of normal liver. They confirm the observations of previous investigators for they show the decidedly favoring influence of an acid medium upon the self-digestion of animal tissue. Autolysis progresses much less rapidly in neutral medium, the amount being in most instances less than one-half that observed in acid. As a rule, a still further reduction occurs in alkaline medium, and in some instances the degree of digestion is almost trivial when compared with the corresponding amount in acid. Experiment VII shows that washing out by perfusion the small amount of serum that remains in the vessels of the liver after exsanguination has practically no influence upon the relative amount of autolysis in acid, neutral and alkaline media. Inasmuch as inhibition of autolysis has been observed in expressed tissue juices after thorough perfusion, it is manifestly impossible to free tissue from all inhibiting substances. Experiments V and VI represent two different types of autolytic activity occurring in normal liver. In both instances what is probably the maximal amount of proteolysis is developed in acid medium. In Experiment V the activity of digestion sinks markedly in alkaline medium, and when a concentration of 0.4 per cent. sodium carbonate is reached, no proteolysis occurs. On the other hand, in Experiment VI a second optimum of digestion occurs in alkaline medium and this high degree of autolytic activity is maintained even in a concentration of 0.4 per cent. sodium carbonate. The latter type of activity is rarely observed in autolysis of normal liver. A similar observation has been reported by Baer and Loeb.[23] Inasmuch as it will be shown later that liver contains a masked alkaline enzyme whose activity can be developed by appropriate methods, the result obtained in Experiment VI suggests that the activity of this enzyme is subject to marked variations during life.

An attempt was made to duplicate, in two additional experiments, the second optimum of activity in alkaline media, which I have described above. Normal liver was allowed to autolyze in strengths of acid and alkali varying from one per cent. acetic acid to one per cent. sodium carbonate.

[23] Baer and Loeb, *loc. cit.*

EXPERIMENT VIII.—The liver of a normal dog with no perfusion was excised aseptically and rapidly hashed. Denaturalized beef serum was added as substrate; the duration of digestion was five days at 37°C.

Quantity of Liver.	Medium.	Digestion.
1.25 gms.	0.6 per cent. acetic acid	24.5 c.c. N/10 H_2SO_4
1.25 gms.	0.4 per cent. acetic acid	23.8 c.c. N/10 H_2SO_4
1.25 gms.	0.3 per cent. acetic acid	22.5 c.c. N/10 H_2SO_4
1.25 gms.	0.2 per cent. acetic acid	21.6 c.c. N/10 H_2SO_4
1.25 gms.	0.1 per cent. acetic acid	19.5 c.c. N/10 H_2SO_4
1.25 gms.	neutral	8.2 c.c. N/10 H_2SO_4
1.25 gms.	0.1 per cent. sodium carbonate	8.3 c.c. N/10 H_2SO_4
1.25 gms.	0.2 per cent. sodium carbonate	2.2 c.c. N/10 H_2SO_4
1.25 gms.	0.3 per cent. sodium carbonate	2.3 c.c. N/10 H_2SO_4
1.25 gms.	0.4 per cent. sodium carbonate	2.5 c.c. N/10 H_2SO_4
1.25 gms.	0.6 per cent. sodium carbonate	3.0 c.c. N/10 H_2SO_4

EXPERIMENT IX.—In this experiment no substrate was added to the autolytic mixtures. Otherwise it resembled Experiment VIII.

Quantity of Liver.	Medium.	Digestion.
2.5 gms.	1.0 per cent. acetic acid	35.9 c.c. N/10 H_2SO_4
2.5 gms.	0.6 per cent. acetic acid	34.3 c.c. N/10 H_2SO_4
2.5 gms.	0.4 per cent. acetic acid	35.6 c.c. N/10 H_2SO_4
2.5 gms.	0.2 per cent. acetic acid	34.8 c.c. N/10 H_2SO_4
2.5 gms.	0.1 per cent. acetic acid	31.5 c.c. N/10 H_2SO_4
2.5 gms.	neutral	11.3 c.c. N/10 H_2SO_4
2.5 gms.	0.1 per cent. sodium carbonate	13.0 c.c. N/10 H_2SO_4
2.5 gms.	0.2 per cent. sodium carbonate	4.8 c.c. N/10 H_2SO_4
2.5 gms.	0.4 per cent. sodium carbonate	0.6 c.c. N/10 H_2SO_4
2.5 gms.	0.6 per cent. sodium carbonate	0.5 c.c. N/10 H_2SO_4
2.5 gms.	1.0 per cent. sodium carbonate	0.7 c.c. N/10 H_2SO_4

The results obtained in these two instances are practically identical. A maximum of digestion is obtained in all grades of acid media, but very slight increase occurs in the higher concentrations of acid. An abrupt fall of activity is manifested in neutral medium. In 0.1 per cent. sodium carbonate the amount of autolysis is somewhat higher than that observed in neutral. When a strength of 0.2 per cent. sodium carbonate is reached, another drop in activity takes place and reduces the degree of autolysis to a very small amount. In Experiment VIII no further reduction occurs on increasing the concentration of alkali; in Experiment IX a concentration of 0.4 per cent. sodium carbonate completely checks autolysis. The only evidence in these two experiments of the pres-

ence of an enzyme acting best in alkaline medium is the equal or slightly increased amount of autolysis that is observed in 0.1 per cent. sodium carbonate.

THE EFFECT OF STANDING UPON THE ENZYMOTIC ACTIVITY OF LIVER.

When fresh normal liver is allowed to stand on ice for considerable periods of time, and its power of autolysis determined at varying intervals, a striking increase of autolytic activity in neutral and alkaline media is observed.

EXPERIMENT X.—In this experiment the liver of a dog poisoned by phosphorus was used. The liver was excised aseptically after being thoroughly perfused with salt solution. It was rapidly hashed and diluted with 0.85 per cent. salt solution. Two flasks were prepared; the liver in one was allowed to stand at neutral reaction, and in the other, in a concentration of 0.1 per cent. sodium carbonate. Both flasks were kept on ice. Sufficient quantities of toluol were added to each flask to inhibit the growth of bacteria. Tests of the autolytic activity in acid, neutral and alkaline media were made immediately. Each autolytic mixture contained 0.5 grams of liver; the duration of digestion was five days at 37°C. Beef serum denaturalized by heat was used as substrate. For this experiment I am indebted to Miss B. I. Barker.

The figures represent cubic centimeters of N/10 sulphuric acid.

Liver kept in 0.85 per cent. salt solution at neutral reaction.

Medium.	Duration of period of standing before tests were made.						
	Fresh.	8 days.	20 days.	29 days.	36 days.	44 days.	51 days.
0.2% acetic acid	11.0 c.c.	13.0 c.c.	10.9 c.c.	11.8 c.c.	10.3 c.c.	10.9 c.c.	9.1 c.c.
neutral	1.9 c.c.	1.4 c.c.	3.5 c.c.	3.2 c.c.	2.3 c.c.	3.4 c.c.	3.6 c.c.
0.2% sodium carbonate	0.5 c.c.	2.2 c.c.	4.0 c.c.	5.4 c.c.	5.0 c.c.	4.9 c.c.	5.1 c.c.

Liver kept in 0.85 per cent. salt solution in 0.1 per cent. sodium carbonate.

Medium.	Duration of period of standing before tests were made.						
	Fresh.	8 days.	20 days.	29 days.	36 days.	44 days.	51 days.
0.2% acetic acid	11.0 c.c.	11.0 c.c.	10.3 c.c.	10.9 c.c.	10.5 c.c.		9.1 c.c
neutral	1.9 c.c.	2.3 c.c.	4.6 c.c.	2.6 c.c.	2.4 c.c.	4.1 c.c.	3.1 c.c.
0.2% sodium carbonate	0.5 c.c.	1.3 c.c.	1.9 c.c.	2.2 c.c.	2.6 c.c.	3.8 c.c.	4.6 c.c.

EXPERIMENT XI.—Healthy normal dog's liver was used for this experiment. The animal was bled to death from the carotid arteries and the liver was not perfused. Otherwise, the same details were observed as in Experiment X. To one part of the liver, however, sufficient sodium carbonate was added to make the concentration 0.2 per cent. For each test of autolysis 2.5 grams of liver were used. The duration of digestion was five days at 37°C., and denaturalized beef serum was used as substrate.

The figures represent cubic centimeters of N/10 sulphuric acid.

Liver kept in 0.85 per cent. salt solution at neutral reaction.

Medium.	Duration of period of standing before tests were made.					
	Fresh.	10 days.	20 days.	31 days.	45 days.	55 days.
0.2% acetic acid	29.4 c.c.	20.9 c.c.	23.7 c.c.	20.7 c.c.	20.7 c c.	17.6 c.c.
neutral	7.9 c.c.	16.3 c.c.	9.9 c.c.	22.9 c.c.	17.9 c.c.	21.7 c.c.
0.2% sodium carbonate	2.0 c.c.	7.6 c.c.	3.1 c.c.	17.4 c.c.	15.0 c.c.	24.2 c.c.

Liver kept in 0.85 per cent. salt solution in 0.2 per cent. sodium carbonate.

Medium.	Duration of period of standing before tests were made.					
	Fresh.	10 days.	20 days.	31 days.	45 days.	55 days.
0.2% acetic acid	29.4 c.c.	16.9 c.c.	17.5 c.c.	15.6 c.c.	16.4 c.c.	15.1 c.c.
neutral	7.9 c.c.	16.6 c.c.	16.5 c.c.	20.9 c.c.	16.7 c.c.	21.6 c.c.
0.2% sodium carbonate	2.0 c.c.	4.0 c.c.	1.2 c.c.	11.0 c.c.	7.4 c.c.	13.0 c.c.

EXPERIMENT XII.—The liver used in this experiment was obtained from a normal dog. The animal was bled to death and the liver was not perfused. The method was the same as in Experiment XI, except that in this instance no substrate was added. In each test of autolysis 2.5 grams of liver were used.

Liver kept in 0.85 per cent. salt solution at neutral reaction.

Medium.	Duration of period of standing before tests were made.		
	Fresh.	15 days.	37 days.
0.2% acetic acid	20.7 c.c. N/10 H_2SO_4	17.7 c.c. N/10 H_2SO_4	14.6 c.c. N/10 H_2SO_4
0.2% sodium carbonate	3.6 c.c.	11.0 c.c.	15.0 c.c.

Liver kept in 0.85 per cent. salt solution in 0.2 per cent. sodium carbonate.

Medium.	Duration of period of standing before tests were made.		
	Fresh.	15 days.	37 days.
0.2% acetic acid	20.7 c.c. N/10 H_2SO_4	10.4 c.c. N/10 H_2SO_4	10.8 c.c. N/10 H_2SO_4
0.2% sodium carbonate	3.6 c.c.	2.3 c.c.	14.6 c.c.

In the above experiments tests of the reaction of the liver solution allowed to stand were made from time to time. The liver

kept in salt solution developed a weak acidity, but in that to which alkali was added immediately, the alkaline reaction was maintained constantly throughout the experiment. At no time was it possible to demonstrate the presence of bacteria; in fact, liver extracts which have been kept on ice for some time have been found to be powerfully bacteriolytic. In all the experiments it is apparent that the addition of acid to the autolytic mixtures of fresh liver develops a maximal activity and the degree of autolysis in acid medium is not increased as the liver stands, but tends rather to diminish slightly. This diminution is more evident in the liver which stands in alkaline solution than in that to which no alkali has been added. Immediate autolysis of fresh liver in neutral and alkaline medium is observed to be relatively low. Upon standing, however, a striking increase in both media occurs until, as is shown in Experiments XI and XII, the degree of autolysis exceeds that observed in acid. The rate of increase of activity is slower in liver kept in alkaline solution than in that kept in neutral medium. The presence of alkali retards but does not prevent the activation of the enzyme acting in alkaline medium. The periods of diminished activity in neutral and alkaline medium observed from time to time are somewhat difficult to explain, but possibly depend upon the development of inhibiting substances during the slow disintegration that proceeds while the liver stands on ice. The increase of autolytic activity in neutral and alkaline medium might be supposed to be due to a slow destruction of the anti-enzyme of the serum, but this destruction is rendered improbable by the observation that the same increase occurs in liver thoroughly perfused, and, moreover, in liver kept continuously in alkaline solution; alkaline reaction not only preserves the serum anti-enzyme, but even increases somewhat its anti-proteolytic power. The increase of proteolytic activity observed in these experiments is analogous to the increase of tryptic activity observed by Vernon[24] in kept pancreatic extracts. This increase Vernon attributes to the slow conversion of trypsinogen into active trypsin. It is probable, therefore, that a like process occurs in kept liver, and normal liver contains two proteolytic enzymes, one acting best in alkaline and the other in acid

[24] Vernon, *Jour. of Physiol.*, 1901, xxvii, 269.

medium. The alkaline enzyme in fresh tissue, however, exists in an inactive state, so that the amount of immediate autolysis in alkali is slight. Upon standing, the inactive enzyme is slowly converted into active enzyme and a corresponding rise in autolytic activity in alkaline medium results. If the acid-acting enzyme exists in the cell also in an inactive form, it is readily activated by the presence of acid and, consequently, even when fresh tissue is used, exhibits its maximal activity in acid medium. It will be shown later in this paper that the enzyme of liver acting in alkaline medium can be activated by treatment with acid. The activating influence of acid upon trypsinogen of fresh pancreas has been known for many years.

THE EFFECT UPON SUBSEQUENT AUTOLYSIS OF PRETREATMENT OF LIVER WITH ACID AND WITH ALKALI.

Hedin [25] has published the interesting observation that treatment of certain animal organs for from sixteen to twenty-four hours with 0.2 per cent. acetic acid increases the degree of subsequent auto-digestion in alkaline medium. This increase he has shown to be due in the spleen to the destruction by acid of an anti-enzyme which holds in check the weak alkali-acting enzyme which is present. In experiments which follow, the methods used by Hedin have been applied with some modifications to autolysis of liver. By these means it has been possible to produce rapidly an increase of the proteolytic activity of the enzyme acting in alkaline medium.

The livers used in the following experiments were obtained from healthy normal dogs. They were excised aseptically and were minced by passing rapidly through a hashing machine. Three flasks of liver were prepared for each experiment and all were diluted with a quantity of physiological salt solution, equal in amount to the number of grams of tissue used. To one flask acetic acid was added until the required concentration was reached, to another sodium hydrate was added to a like concentration, and the third flask was kept at neutral reaction. All the mixtures were allowed to stand for twenty-four hours on ice, and then the acid flask was neutralized with N/10 sodium hydrate and the alkaline

[25] Hedin, Festschr. f. Olof Hammarsten, Upsala, 1906, vi, 1.

flask with acetic acid. Corresponding amounts of sodium hydrate and acetic acid were not added to the neutral flask because the resulting salt was found to exercise no influence upon autolysis. After neutralization equal amounts of the liver mixtures were withdrawn from each flask and allowed to autolyze in the thermostat in acid, neutral, and alkaline media. In the earlier experiments denaturalized beef serum was added to each flask as substrate, but its use was later discontinued. Toluol was used in all experiments as an antiseptic.

EXPERIMENT XIII.—The liver was obtained from a normal dog bled to death from the carotid arteries. It was excised aseptically without perfusion and passed rapidly through a hashing machine. The liver was prepared for pretreatment as follows: 5 grams of liver + 20 c.c. salt solution; 5 grams of liver + 15 c.c. salt solution + 5 c.c. 1 per cent. acetic acid; 5 grams of liver + 15 c.c. salt solution + 5 c.c. 1 per cent. sodium hydrate.

All the flasks were allowed to stand for twenty-four hours on ice, when the acid and alkali were carefully neutralized, and portions of liver from each flask allowed to autolyze in acid, neutral, and alkaline media for five days at 37°C. Denaturalized beef serum as substrate was used in each mixture. Each flask contained one gram of liver. The figures given represent the amount of digestion after the subtraction of controls.

Medium.	Treated with 0.2% acetic acid.	Untreated.	Treated with 0.2% sodium hydrate.
0.2% acetic acid	14.3 c.c. N/10 H_2SO_4	16.6 c.c. N/10 H_2SO_4	7.0 c.c. N/10 H_2SO_4
Neutral	6.1 c.c. N/10 H_2SO_4	4.3 c c. N/10 H_2SO_4	1.4 c.c. N/10 H_2SO_4
0.2% sodium carbonate	4.7 c.c. N/10 H_2SO_4	3.4 c.c. N/10 H_2SO_4	1.0 c.c. N/10 H_2SO_4

EXPERIMENT XIV.—The liver of a normal dog was excised aseptically and washed by being thoroughly perfused with salt solution. The liver was prepared for pretreatment as follows: 10 grams of liver + 20 c.c. salt solution; 10 grams of liver + 17.6 c.c. salt solution + 2.4 c.c. 5 per cent. acetic acid; 10 grams of liver + 17.6 c.c. salt solution + 2.4 c.c. 5 per cent. sodium hydrate.

The flasks were allowed to stand for twenty-four hours on ice. The acid and alkali were then carefully neutralized and the liver allowed to autolyze in acid, neutral, and alkaline media with denaturalized beef serum as substrate. The duration of digestion was five days at 37°C. Each flask contained 2.5 grams of liver. The controls have been subtracted from the amounts of digestion noted.

Medium.	Treated with 0.4% acetic acid.	Untreated.	Treated with 0.4% sodium hydrate.
0.4% acetic acid	27.7 c.c. N/10 H_2SO_4	37.3 c.c. N/10 H_2SO_4	1.3 c.c. N/10 H_2SO_4
Neutral	15.6 c.c. N/10 H_2SO_4	10.5 c.c. N/10 H_2SO_4	10.4 c.c. N/10 H_2SO_4
0.2% sodium carbonate	5.6 c.c. N/10 H_2SO_4	1.5 c.c. N/10 H_2SO_4	9.5 c.c. N/10 H_2SO_4

From the liver used in this experiment another series of flasks was prepared in the manner described above. Instead of standing only twenty-four hours at the various reactions, they were allowed to stand for six days before neutralization.

Medium.	Treated with 0.4% acetic acid.	Untreated.	Treated with 0.4% sodium hydrate.
0.4% acetic acid	26.1 c.c. N/10 H_2SO_4	37.1 c.c. N/10 H_2SO_4	0.7 c.c. N/10 H_2SO_4
Neutral	16.3 c.c. N/10 H_2SO_4	15.4 c.c. N/10 H_2SO_4	6.5 c.c. N/10 H_2SO_4
0.2% sodium carbonate	13.5 c.c. N/10 H_2SO_4	3.2 c.c. N/10 H_2SO_4	8.1 c.c. N/10 H_2SO_4

Experiment XV.—Normal dog's liver was excised aseptically and rapidly hashed. Two series of flasks were prepared. In one series the liver contained what blood remained in the vessels after bleeding, and in the other the cells were carefully washed by centrifugalization with physiological salt solution. Each series consisted of two flasks, the liver in one of which was treated with 0.2 per cent. acetic acid for twenty-four hours on ice, and the other allowed to stand for the same period of time at neutral reaction. The acid flasks were neutralized with N/10 sodium hydrate before the preparation of the autolytic mixtures. Each flask of the latter contained five grams of liver. No substrate was added. The controls have been subtracted. The lower degree of autolysis observed in the washed cells is dependent upon the loss of liver enzyme during the process of washing and centrifugalization.

Unwashed cells.

Medium.	Treated with 0.2% acetic acid.	Untreated.
0.2% acetic acid	45.4 c.c. N/10 H_2SO_4	49.9 c.c. N/10 H_2SO_4
Neutral	31.1 c.c. N/10 H_2SO_4	16.4 c.c. N/10 H_2SO_4
0.2% sodium carbonate	19.7 c.c. N/10 H_2SO_4	17.9 c.c. N/10 H_2SO_4

Washed cells.

Medium.	Treated with 0.2% acetic acid.	Untreated.
0.2% acetic acid	20.2 c.c. N/10 H_2SO_4	26.1 c.c. N/10 H_2SO_4
Neutral	13.3 c.c. N/10 H_2SO_4	12.7 c.c. N/10 H_2SO_4
0.2% sodium carbonate	16.7 c.c. N/10 H_2SO_4	10.2 c.c. N/10 H_2SO_4

Experiment XVI.—The liver was obtained from a normal dog. It was excised aseptically and hashed without being perfused. Flasks were prepared for pretreatment as follows: 10 grams of liver + 20 c.c. salt solution; 10 grams of liver + 17.6 c.c. salt solution + 2.4 c.c. 5 per cent. acetic acid; 10 grams of liver + 17.6 c.c. salt solution + 2.4 c.c. 5 per cent. sodium hydrate.

All flasks were allowed to stand twenty-four hours on ice, after which the acid and alkali were neutralized. The liver was allowed to autolyze for five days at 37°C. No substrate was added. Each autolytic mixture contained 2.5 grams of liver. The controls have been substracted.

Medium.	Treated with 0.4% acetic acid.	Untreated.	Treated with 0.4% sodium hydrate.
0.2% acetic acid	22.5 c.c. N/10 H_2SO_4	34.8 c.c. N/10 H_2SO_4	3.9 c.c. N/10 H_2SO_4
Neutral	17.2 c.c. N/10 H_2SO_4	12.8 c.c. N/10 H_2SO_4	0.4 c.c. N/10 H_2SO_4
0.4% sodium carbonate	16.8 c.c. N/10 H_2SO_4	7.7 c.c. N/10 H_2SO_4	0.1 c.c. N/10 H_2SO_4

EXPERIMENT XVII.—A normal dog was bled to death from the carotid arteries. The liver was used without being perfused. The flasks were prepared for pretreatment as follows: 10 grams of liver + 20 c.c. salt solution; 10 grams of liver + 17.6 c.c. salt solution + 2.4 c.c. 5 per cent. acetic acid; 10 grams of liver + 17.6 c.c. salt solution + 2.4 c.c. 5 per cent. sodium hydrate.

All flasks stood for twenty-four hours on ice. The acid and alkali were then neutralized and the autolytic mixtures prepared. The duration of digestion was five days at 37°C. No substrate was added. This liver was especially active in alkali when fresh, so that it was impossible to increase to any degree the alkaline activity by acid treatment. Figures representing digestion are given both before and after the subtraction of controls in order to show how the acid-treated liver may apparently exhibit no increase of activity because a high control must be deducted from the observed amounts of digestion.

The figures represent cubic centimeters of N/10 sulphuric acid.

Medium.	Treated with 0.4 per cent. acetic acid.		Untreated.		Treated with 0.4 per cent. sodium hydrate.	
	Digestion observed	Same with control subtracted.	Digestion observed.	Same with control subtracted.	Digestion observed.	Same with control subtracted.
0.2 per cent. acetic acid	25.5 c.c.	14.8 c.c.	28.6 c.c.	23.2 c.c.	6.9 c.c.	0.9 c.c.
Neutral	20.9 c.c.	10.2 c.c.	15.2 c.c.	9.8 c.c.	8.8 c.c.	2.8 c.c.
0.2 per cent. sodium carbonate	24.7 c.c.	14.0 c.c.	18.9 c.c.	13.5 c.c.	9.2 c.c.	3.2 c.c.
0.4 per cent. sodium carbonate	21.4 c.c.	10.7 c.c.	13.6 c.c.	8.2 c.c.	7.4 c.c.	1.4 c.c.

These experiments are in agreement with the experiments of Hedin[20] upon the effect of acid treatment upon autolysis of spleen. In every instance treatment of liver with weak acetic acid has markedly increased the amount of subsequent autolysis in sodium carbonate. The increase of autolysis in alkali is in most instances paralleled by a like increase in neutral medium. The degree of increased activity obtained is dependent upon the initial activity in alkali of the liver when fresh and upon the duration of the time of treatment. Pretreatment of fresh liver with alkali differs from like treatment with acid for it results in the destruction of practically all proteolytic power, whatever the subsequent medium of

[20] Hedin, *loc. cit.*

digestion may be. The increase of activity produced by acid treatment is not entirely due to the destructive action of acid upon the anti-enzymotic power of any serum that may remain in the tissue, for in Experiment XV increase of autolysis in alkaline medium observed in cells thoroughly washed in salt solution is somewhat greater than in those cells which have not been washed. The addition to the autolytic mixtures of denaturalized beef serum causes inhibition of autolysis in alkaline medium. To this thermostabile inhibitory power is attributable the comparatively low degrees of alkaline digestion after acid treatment of liver observed in those experiments in which heated serum has served as substrate. In acid medium, on the other hand, denaturalized serum is actively attacked.

In discussing the increased activity of acid-treated liver in alkaline medium, two other factors must be considered. It has been mentioned that the degree to which the activity of liver in alkali can be increased by treatment with acid is dependent upon the power of the liver to undergo self-digestion in alkali when fresh. Another factor which diminishes the apparent activity produced by acid treatment is the necessity of subtracting a large control referable to autolysis which takes place on ice during pretreatment with acid. Both of these factors are well illustrated in Experiment XVII. The liver in this experiment is more active in alkaline medium than any that has been studied. Consequently, the increase of activity in alkali produced by acid treatment is comparatively small, and because the difference between the controls of the acid-treated and the untreated liver is large, entirely disappears when the figures representing controls are subtracted from those representing digestion. Such active livers are, however, rarely observed, and the commonest type of activity is illustrated by Experiment XVI, in which acid-treatment more than doubles the amount of subsequent autolysis in sodium carbonate.

There is some reason to believe that the inactivity following pretreatment of liver with 0.4 per cent. sodium hydrate is not referable to the complete destruction of the proteolytic enzyme, but is due to the fixing of the enzyme in the inactive state in which it

exists within the liver cells. In Experiment XIV the liver is fairly active in neutral and alkaline media after treatment with alkali, but shows no activity in acid medium after the treatment. This has been observed in a few other experiments and the possibility that the heated serum added in this instance contained a substance capable of activating the alkaline enzyme of liver was considered, but attempts to confirm this observation were negative. In fact, all attempts to reactivate the proteolytic enzymes of liver after alkaline treatment have failed. When fresh pancreas is treated with sodium hydrate there is the same inactivity observed as after like treatment of liver, but this inactive pancreatic extract can readily be activated by the addition of enterokinase. This fact will be more fully discussed in a future publication. Furthermore, when liver which has become active in alkaline medium by standing is treated with 0.4 per cent. sodium hydrate, inactivity, which occurs when fresh liver is so treated, is not produced.

From the experiments detailed above, it is obvious that the effects of acid and of alkali treatment on fresh liver are distinctly opposite, the former increasing the proteolytic activity in neutral and alkaline media, whereas the latter diminishes or totally abolishes activity in all media. The power of alkali to decrease autolysis can scarcely be termed an inhibition of active enzyme but must be looked upon as a preservation of the enzyme which acts in alkaline medium in an inactive condition. When this enzyme has been activated, for instance, by standing or by acid treatment, weak concentrations of alkali form a favorable medium for its action.

In contrast to the effect of alkaline treatment upon the proteolytic enzymes of liver and pancreas is its effect upon the alkaline enzyme of the polymorphonuclear leucocyte of inflammatory exudates. The following experiment shows that leucoprotease maintains its activity when kept continuously in alkaline medium and is able to effect proteolysis after treatment with sodium hydrate.

EXPERIMENT XVIII.—Leucocytes were obtained by injecting sterile aleuronat into the pleural cavity of a dog. The resulting exudate was centrifugalized and the cells carefully washed with salt solution. One part was treated with 0.2 per cent. acetic acid for twenty-four hours on ice another was allowed to stand in neutral medium, and a third, in 0.2 per cent. sodium hydrate for the same period

of time. The acid and alkali were then carefully neutralized and the digestive power of the cells determined in acid, neutral, and alkaline media. Denaturalized beef serum was used as substrate. The same quantity of cells was employed for each test. The controls have been subtracted.

Medium.	Treated with 0.2% acetic acid.	Untreated.	Treated with 0.2% sodium hydrate.
0.2% acetic acid	10.9 c.c. N/10 H_2SO_4	12.3 c.c. N/10 H_2SO_4	1.2 c.c. N/10 H_2SO_4
Neutral	9.5 c.c. N/10 H_2SO_4	12.8 c.c. N/10 H_2SO_4	8.7 c.c. N/10 H_2SO_4
0.4% sodium carbonate	9.7 c.c. N/10 H_2SO_4	9.7 c.c. N/10 H_2SO_4	5.6 c.c. N/10 H_2SO_4

In this experiment acid treatment has not increased the power of subsequent digestion in neutral medium or in sodium carbonate. Pretreatment with 0.2 per cent. sodium hydrate does not destroy proteolytic activity later tested in neutral and alkaline medium, although the corresponding activity of liver and pancreas so treated is destroyed. The digestion observed in acid medium is referable in part to the activity of the mononuclear cells which Opie[27] has shown contain an enzyme acting in acid medium. Inasmuch as this enzyme is very sensitive to alkali, it is almost completely destroyed by treatment with sodium hydrate.

Experiment XIX.—The leucocytes obtained from a sterile abscess produced by turpentine were washed as in the previous experiment. One part was allowed to stand in neutral medium and another in 0.4 per cent. sodium hydrate for twenty-four hours on ice. The alkali was then neutralized and the digestive power of the mixtures determined as before. No substrate was added. Each flask contained an equal quantity of cells.

Medium.	Untreated.	Treated with 0.4% sodium hydrate.
0.2% acetic acid	4.7 c.c. N/10 H_2SO_4	1.3 c.c. N/10 H_2SO_4
Neutral	6.2 c.c. N/10 H_2SO_4	7.1 c.c. N/10 H_2SO_4
0.2% sodium carbonate	8.1 c.c. N/10 H_2SO_4	4.9 c.c. N/10 H_2SO_4

In this instance, even though the concentration of alkali was increased to 0.4 per cent., the cells were later able to effect proteolysis in neutral and alkaline media.

From these experiments it appears that leucoprotease is different from the alkaline enzymes of liver and pancreas, for it exists in the cells of inflammatory exudates in an active condition. This

[27] Opie, *Jour. of Exper. Med.*, 1906, viii, 410.

assumption is based upon observations of the effect of alkali upon the proteolytic enzymes of liver and pancreas. It has been demonstrated that alkali tends to preserve the enzyme in the inactive condition, and pretreatment with sodium hydrate renders permanent this inactivity; it is noteworthy that pancreas can be activated subsequently by a specific substance, enterokinase of succus entericus.

The work described may be summed up in a few general conclusions. Autolysis of fresh normal liver progresses much more favorably in acid than in alkaline medium. When fresh liver is placed on ice in neutral and in alkaline solution, the power of autolysis in neutral and alkaline media, when tested from week to week, increases markedly, whereas autolysis of the same mixtures in acid medium decreases somewhat. The addition of acid to fresh liver develops the maximal autolytic activity in acid medium. Pretreatment of fresh normal liver with weak acetic acid increases the amount of subsequent autolysis in neutral and alkaline media. The enzyme of liver which is capable of proteolysis in alkaline medium probably exists in the cell in an inactive state and requires activation for the development of its maximal activity. If the acid enzyme of liver exists in an inactive condition, it is readily activated by acid which is the best medium for its action. Dog's liver probably contains two proteolytic enzymes, one acting best in acid and the other in alkaline medium. The inhibitory effect of alkali upon liver autolysis is probably referable to the preservation of the inactive state of the alkaline enzyme. The maintenance of the normal equilibrium of the proteolytic enzymes of the liver cell must be intimately dependent upon the preservation of tissue neutrality; inasmuch as we know, on the one hand, that acid reaction means increased activity even if the acidity is subsequently overcome, and, on the other hand, increase of alkaline reaction causes retardation of proteolysis by preventing the activation of the alkaline enzyme. The observation that leucoprotease exhibits proteolytic activity after pretreatment with sodium hydrate suggests that this enzyme exists in an active state in the cells of inflammatory exudates.

I wish to express my thanks to Dr. E. L. Opie for his suggestions and for his interest in the progress of this work.

THE CELL CHANGES IN AMAUROTIC FAMILY IDIOCY.[1]

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Plates LXI–LXV.

The morbid anatomy of amaurotic family idiocy is now as safely established as are the clinical symptoms of this interesting disorder of early childhood. The changes in cell structure are as typical of this disease as are the mental defect, the marasmus, the blindness associated with the "cherry-red spot in the region of the macula lutea," and the family disposition.

Great progress has been made in the recognition of the pathology of the disease during the past twenty-three years, since one of us first thought of the disease as an arrest of cortical development (agenesis corticalis).[2] Eleven years later, following the researches of Hirsch[3] and others, the disease was seen to depend upon involvement of the gray matter of the entire central nervous system, and still more recently, owing particularly to the work of Schaffer, which we are able happily to corroborate, amaurotic family idiocy has gained additional importance in view of the extraordinary and constant changes in ganglion cell structure which it reveals. Incidentally, as Sachs has pointed out at the meeting at Budapest, this gravest and most fatal of all family diseases is a purely cellular disease. It is of no little moment to have established this one fact. It is the starting point for the future study of the pathology of other hereditary and family diseases of the nervous system.

Schaffer, Hirsch, Sachs, Spielmeyer and others, have insisted that in amaurotic family idiocy not a single ganglion cell escapes, and the cell changes are the same whether the cell examined be in

[1] Received for publication June 7, 1910.

[2] Sachs, *Jour. of Nerv. and Ment. Dis.*, 1887, xiv, 541.

[3] Hirsch, *Jour. of Nerv. and Ment. Dis.*, 1898, xxv, 538.

the cortex, in the basal ganglia, in the spinal cord or in the spinal ganglia or retina.

Our present contribution is to be devoted to an account of these morbid cell structures as revealed by the study of a typical case of amaurotic family idiocy, complicated by rickets.

A child, L.G., aged 2 years, was admitted to Mount Sinai Hospital, May 25, 1908. Through the couresy of Dr. Koplik it remained under observation until its death, June 5, 1908. Two other children of the same family had died of amaurotic family idiocy; one child had died of colitis. There is one healthy child 6 years old.

Our own patient had not passed through any of the infectious diseases of childhood. The parents stated that when the child was one year old, having the fate of the two other children in mind, it was noticed that the child did not develop normally. It did not hold up its head, would start suddenly on hearing noises (hyperacusis), and its vision evidently became defective. The parents noticed constant oscillation of the eyeballs and frequent twitchings of the extremities. They also observed a rapidly increasing emaciation.

At the time of the first examination, May 25, the general physical condition was poor, the head somewhat hydrocephalic and in great disproportion to the size of the body. The child would lie entirely listless until subjected to examination. Any attempt to move it would cause a stiffening and trembling of the arms and legs. The child was violently startled by the slightest clapping of the hands near its bed. The eyes were staring and showed marked rotatory and lateral nystagmus. It was evidently blind; its cry was feeble; respirations were fairly regular except for short intermissions. The anterior fontanelle was open, permitting two fingers; the occipito-frontal circumference was 50.5 cm. The child was wholly unable to sit up or to hold up its head.

As for the general condition, it was noted that there was a general morbiliform eruption over the trunk and exteremities. The right epitrochlear and the left axillary glands were slightly enlarged. There was also slight epiphyseal enlargement of the bones. There was normal development of the teeth considering the age of the child. A marked rachitic rosary was noted. The lungs, heart and liver were normal; the spleen palpable. The extremities were, as a rule, in a tetanoid position; the knee-jerks exaggerated; there was no Babinski. Plantar stimulation caused marked trembling of the entire lower extremity. There was no Kernig symptom. Examination of the fundus revealed the "cherry-red spot" in each eye. The disks were blue in color and sharply outlined.

The child's condition remained practically the same during the period of observation except for the progressing emaciation. At various periods during its stay in the hospital the temperature varied from 98.4° to 102.4°. Without any marked change in the symptoms, and without the occurrence of convulsions, the child died on June 5. The autopsy was performed within a few hours after death by Dr. I. Strauss.

Disregarding all macroscopical changes in the brain, and without heeding the question whether or not minor changes in fissuration are of any importance, we pass to a consideration of the morbid process as it affects the ganglion cells of the central nervous system. It has been intimated before that the pathological process is essentially the same whether the cell examined be from a section of the cortex, of the cerebrum or of the cerebellum, or from a section of the spinal cord. The most striking evidence of degeneration is found in the enlargement of the cell-body. The normal concave contour disappears and the circumference of the cell becomes well rounded. The Nissl substance in the body of the cell begins to disappear; it does not disintegrate but undergoes a gradual absorption. In the vicinity of the nucleus the Nissl bodies can be recognized for a considerable period of time, but in the end they disappear there also, as well as from the dendrites. At first the cell appears to contain a finely granular detritus, which is probably the remains of the disintegrated endocellular network. As the morbid process continues the cell-body is converted into a homogeneous mass.

In frozen sections stained with scarlet R. the ganglion cells take on a red stain, which indicates that the degeneration has resulted in the formation of some lipoid substance. So far as we know, this reaction has not been previously noted. The nucleus is displaced to the margin of the cell at an early period of the disease. In the pyramidal cells of the cortex it is found almost invariably at the base of the apical dendrite. As a rule, the nucleus is irregular in outline, somewhat shrunken, and contains a distinct nucleolus, but very little chromatin. It remains recognizable within the degenerating cell until the final disintegration has occurred.

Next to the degeneration of the cell-body, we are struck by the peculiar balloon-like swelling of the dendrites, to which Shaffer was the first to call special attention. This enlargement may be said to be pathognomonic of the disease. The swelling of the dendrites is especially characteristic of the ganglion cells of the cerebral cortex, as well as of the cells of Purkinje in the cerebellum. In the former the basal dendrites are invariably affected. They may be so dilated as to be considerably larger than the cell-body. The swelling of the

dendrites of the Purkinje cells is also very marked. This same change is found to a lesser degree in the ganglion cells of the spinal cord. In striking contrast to this is the fact that the apical dendrites of the pyramidal cells and the axones are rarely affected. We have no theory as yet to account for this peculiar selective activity of the degenerative process (Plate LXI, Figs. 1 and 2; Plate LXII, Figs. 3 and 4).

In his excellent study on amaurotic family idiocy, Schaffer[4] observes that "Sachs' disease may be diagnosticated by the appearance of the fibrillæ." We cannot subscribe fully to this statement. Somewhat similar changes in the fibrillæ, their disappearance in the cell-body and their persistence in the dendrites and axone have been described in other diseases, as shown by Goldstein's[5] findings in dementia præcox. Before entering upon the description of the changes in the fibrillæ of the ganglion cells of the central nervous system in amaurotic family idiocy, it will be best to review briefly the present-day knowledge of neurofibrils. . . . Within the cell-body, but very near its surface, is a well-defined network of fibrils. This network is also present in the dendrites and in the axone. It is called the epicellular or ectocellular network of neurofibrils. This network receives fibrils from beyond the cell, and by some it is thought to be connected with a finer fibrillary network occupying most of the cell-body, and known as the endocellular network. The meshes of the latter network are smaller, and around the nucleus it is denser in appearance. This denser part is designated as the pericellular network.[6]

Bethe describes the epicellular network, which he considers the same as the Golgi net, but denies that there is any endocellular network. He claims that the fibrils pass through the cell without forming anastomoses. Donaggio, however, with a slightly different method has demonstrated an endocellular network. Cajal, and many of those using the silver method, describe primary fibrils

[4] *Zeit. f. d. Erforsch. u. Behandl. d. jugendlichen Schwachsinns,* 1909, iii, 53.

[5] *Arch. f. Psychiat.,* 1910, xliv, 1062.

[6] The principal studies on neurofibrils have been made by Bethe and Donaggio, using a method which depends upon the action of ammonium molybdate upon methylene blue. Cajal and Bielschowsky have pursued the same researches, relying upon a method of silver precipitation.

which are thick and pass through the cell but at the same time anastomose with a finer set of fibrils which form a dense network about the nucleus. In order to harmonize the views of Bethe and Cajal, Economo, a pupil of Bethe, working under his supervision and studying the same materials by the various methods, concluded that the epicellular network is a well-defined structure connected with the fibrils of the cell-processes and that it is also frequently connected with fibrils coming from beyond the cell. In his opinion the thickening of the primary fibrils of Cajal and the appearance of interlacing are due to the silver nitrate's causing the fibrils to shrink and to be approximated to each other, thus simulating a network. According to Economo the endocellular network is due to thorough impregnation of the protoplasmic reticulum. The isolated fibrils lie in this reticulum. He and Bielschowsky believe that the isolated fibrils run over and pass the nucleus and that the perinuclear network is an artifact due to the method of impregnation.

In his first studies of the fibrils in amaurotic idiocy Schaffer described an outer network of coarse anastomosing fibrils which he considered identical with the Bethe-Golgi net, or the epicellular network of Economo. Schaffer also described an inner network which he regarded as equivalent to the endocellular network of Donaggio. He observed that the outer network of fibrils is very evident in the dendritic processes and that the fibrils anastomose with each other and are connected with the inner network. Bielschowsky questioned this connection between the two networks and claimed, in agreement with Economo, that the inner was an artifact and was caused by the impregnation of a protoplasmic reticulum. In his latest work Schaffer still claims that his preparation showed the presence of the two networks, but because the endocellular one has the staining characteristics which Economo and Bielschowsky ascribe to the protoplasmic reticulum, and because of its behavior in the degenerating cell, he agrees with the writers just mentioned in the observation that this is not a true neurofibril structure.

We find that the pyramidal cells of the cortex, the Purkinje cells, and the large anterior horn cells, which we have studied by Bielschowsky's method, show the epicellular network very distinctly

(Plate LXIII, Fig. 5). The fibrils are seen in the dendrites as parallel strands closely approximated and freely anastomosing with one another. This anastomosis may be due to the blending or shrinking together which Economo claims gives rise to this appearance in tissues treated by Cajal's method, but in sections in which the dendrite is characteristically swollen one can often see the fibrils separated by a considerable space from one another and also having branches which unite them. The epicellular fibrils from the dendrites can be traced into the cell-body where they form a superficial network (Plate LXIII, Fig. 6). Schaffer has pointed out that there is a distinct network of strands of still finer calibre occupying the greater part of the cell-body within the endocellular network. Around the nucleus this network appears to have more numerous strands and finer meshes so that it has a denser appearance. This denser network forms the perinuclear network. We have observed the fibrils from the epicellular net of the dendrites passing over and around the nucleus, apparently not forming any connection with the perinuclear net. We have also observed the fibrils from the dendrites passing directly into the perinuclear net and joining the endocellular neurofibrils. The neurofibrils in the axone usually form a single black strand. Frequently they can be seen entering the cell-body, spreading out in funnel-fashion, and merging into the perinuclear network. We have not been able to corroborate Economo's description of the color characteristic of the endocellular network. The epicellular fibrils differ from the endocellular in being stouter and more deeply stained, also more permanent. It would seem that the endocellular network was the first to show evidences of degeneration (Plate LXIII, Fig. 7; Plate LXIV, Fig. 8). The fibrils become granular and often disappear leaving a granular detritus. The perinuclear network remains for a considerable period of time, but even after the cell has become a large, swollen, balloon-like structure, and after the nucleus has become either degenerated or has entirely disappeared, the dendrites may still remain and show the presence of fibrils (Plate LXIV, Fig. 9). The last of all the cell structures to disappear is the axone with its fibrils. Schaffer argued originally that the early disappearance (Plate LXIV, Fig. 10) of the endocellular neurofibrils proved that

they were not functionally an essential part of the cell, but that the interfibrillary substance must be the conducting element.

According to his latest views, Karl Schaffer is inclined to believe that the morphological endocellular network is connected with the epicellular net, but since it disappears very early in cells which still retain fibrils in the dendrites and in the axone, it does not represent a true neurofibril structure. This conclusion of Schaffer's does not seem wholly warranted. It is nothing unusual to find a structure which is more vulnerable in one part than in another, and for this reason alone we are not compelled to assume that there is any structural difference between these parts. In a nerve which has been cut the degeneration of the fibrils in the proximal part proceeds to Ranvier's node, and yet no one for this reason would assume that there is any difference in the structure of the axone fibrils at any part of their course. If Schaffer's contentions were correct, we should be compelled to assume that the fibrils and the dendrites of the axone were different in character, because the former disappear in the degenerating cell while the latter remain.

The most important conclusions to be drawn from a study of the fibrils of the ganglion cells of amaurotic idiocy are (1) that the endocellular network is most susceptible to the degenerative process, and since there is good reason to believe that the cells preserve their function in the earlier stage of degeneration, these fibrils cannot be the conducting elements, and (2) we must note the remarkable independence of the axone and its fibrils from the rest of the cell. This is shown not only by the persistence of the axone near the cell, but by the absence of degenerated fibers in the cord.

Schaffer was the first to describe the presence in the cortex of round structureless cells with the nucleus rich in chromatin. He noted that they were found in fewer numbers in the white matter directly under the cortex. He thought them glia cells undergoing retrogressive metamorphosis. In his latest article he refers to Eisarth's studies on "The Histology of the Human Neuroglia," in which such cells are described as being characteristic of changes in the glia in the degenerative psychoses of early life. We have noticed these peculiar cells in large numbers both in the cerebral and in the cerebellar cortex (Plate LXV, Fig. 11). They are also

fairly abundant in the subcortical white matter. Their cytoplasm, however, is not structureless as Shaffer thought, but when the cells are stained with scarlet R. they are found to be filled with fat droplets. The nuclei vary in size from those of the normal glia cells to one considerably smaller and more deeply stained, at least in those cells which are filled with fat. These cells frequently occur in nests and can often be seen not only in the vicinity of the degenerating ganglion cells, but they also actually invade them (Plate LXV, Fig. 12). They appear to play the rôle of neuronophagocytes. These cells, it is interesting to observe, are also found in considerable numbers in the perivascular spaces of the vessels of the cortex Plate LXV, Fig. 13) and also in the subpial space over the surface of the cortex (Plate LXV, Fig. 14).[7]

There can be no doubt that these peculiar cells, which we agree with Schaffer in interpreting as altered glia cells, bear a striking resemblance to the granular cell which is found in brain tissue bordering upon an irritating lesion. In the brain of the amaurotic idiot the presence of these granular cells is explained by the degeneration of the ganglion cell acting as an irritant to the surrounding tissue. There is no reason to claim that the morbid process is at all inflammatory in character.

The glia shows an increase of fiber growth in the cortex and in the antero-lateral ground bundle and direct pyramidal tract region of the cord. This growth may be partly of a substantive nature which is due to the degeneration of the cortical ganglion cells and partly to the inhibited development of the motor paths in the cord. Possibly some of this growth is due to the same exciting cause which is responsible for the process of degeneration.

In several previous publications Sachs called attention to the striking absence of the degeneration of the white fibers in the brain and spinal cord. In the present research this same fact has been observed. There is a twofold reason for this: first, as has been shown by Schaffer and by us, the axone remains apparently unaffected by the disease process, and, secondly, we must suppose that some, if not many, of the cells retain some trophic influence

[7] We are indebted to Dr. F. S. Mandlebaum, Pathologist of Mount Sinai Hospital, New York, for the photographs reproduced in this article.

over the peripheral fibers in spite of the marked change in morphological structure. Such slight changes as have been noted by us and others in the pyramidal tracts are not due to a true degeneration, but are the expression of imperfect myelinization. As is well known, the fibers of the pyramidal tracts are the last to acquire their myelin sheaths; hence it is not surprising that they are less deeply stained than are the other spinal tracts at this early period of life.[8]

The attempt to interpret the pathological findings in amaurotic family idiocy has led to an apparent conflict of opinion regarding the origin of the disease. In previous publications one of us defended the theory of an arrest of development affecting the ganglion cells. The thought that the disease was due to an *agenesis corticalis* was abandoned a number of years ago, after Hirsch had found that it was not the gray matter of the cortex, but the gray matter of the entire central nervous system that was involved in this disease.

The theory of an arrest of development has to be given up since embryologists limit this term to the conception of an arrest due to the cessation of the normal morphological growth, whereas so far as we know the brain of the amaurotic idiot is morphologically normal at birth and for some time after. Others must abandon the idea that so strictly a family disease can be due to a definite infectious or toxic agent acting from without.

Amaurotic family idiocy is unique among congenital diseases because of the specific and pathognomonic change in the ganglion cells, and of the involvement of every part of the nervous system. The gray matter of the brain and spinal cord is morphologically well formed and, so far as we know, functionally normal up to the age

[8] Through the courtesy of Dr. Spielmeyer we have been privileged to study some of the sections from his case of the juvenile form of amaurotic idiocy. While there is a superficial resemblance between the cell changes in these two varieties of amaurotic idiocy, the differences are still more striking. In the juvenile form, the disease process is not as universal as in the infantile form; there are many relatively normal cells, the cell contours are not completely obliterated and we fail to find the typical balloon-like enlargement of the cell bodies and the swelling of the dendrites which are so characteristic of the cells in the Tay-Sachs type.

of four or six months. Then there gradually occurs a degeneration of all the ganglion cells and a consequent loss of function. The tendency to this degeneration must be born with the child and must reside in the germ plasm. Unfortunately, our knowledge of the laws of heredity as yet furnishes us with no idea as to the nature of this inherited taint. It is, however, not sufficient merely to predicate an impairment of the germ plasm; this would not explain the normal development of the nervous system through the early months of infancy and its degeneration a few months later. We must suppose that at about the fourth or sixth month some endogenous factor, related to the metabolic processes of the body, intervenes and affects the cells which have inherited a lessened resistance to this agent. The factor may be one which is present in all children but does not affect the nervous system of those who are not born with this inherited weakness.

Hirsch supposed that the change was due to an infection conveyed through the mother's milk, but his theory has been positively disproved by the fact that several of the children described by Sachs and Schaffer have not been nursed by their own mothers, and inasmuch as several children in the same family have been raised by different nurses, this theory falls absolutely to the ground. Poynton, Parson and Holmes, who have opposed Sachs's original idea of an arrest of development, are convinced that "The disease is due to some inherent bio-chemical property of the protoplasm of the cells." After all, the "bio-chemical property" is "inherent" in the cell, which is equivalent to saying that there is something in the cell born with it and not acquired by it. An additional factor must be assumed to explain the late appearance and the specificity of the degeneration.

EXPLANATION OF PLATES.

PLATE LXI.

FIG. 1. Pyramidal cells from the cortex. Balloon-like swelling of body and basal dendrites, displacement of nucleus to base of apical dendrite, degeneration and disappearance of Nissl substance. × 560.

FIG. 2. Anterior horn, spinal cell. Enlargement, balloon-like (?), degeneration, displacement of nucleus, remains of Nissl substance. × 280.

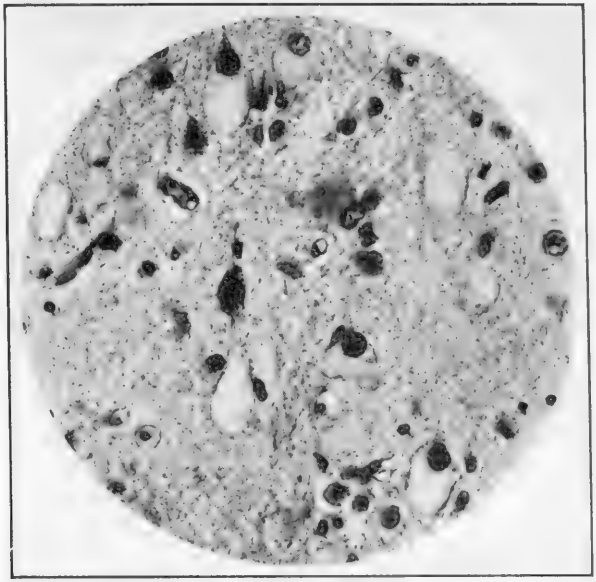


FIG. 1.

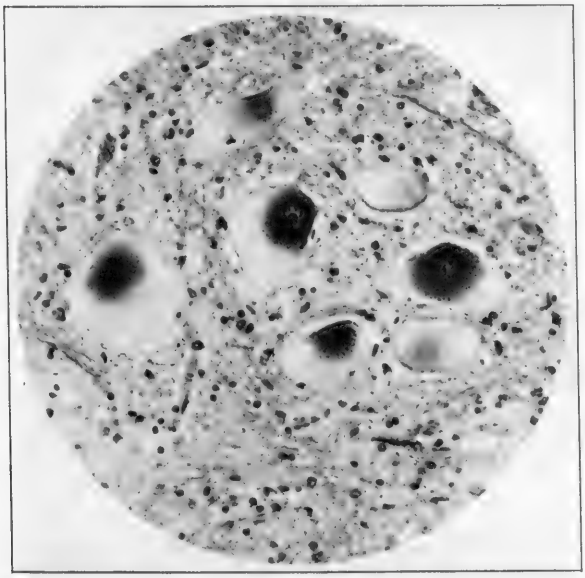
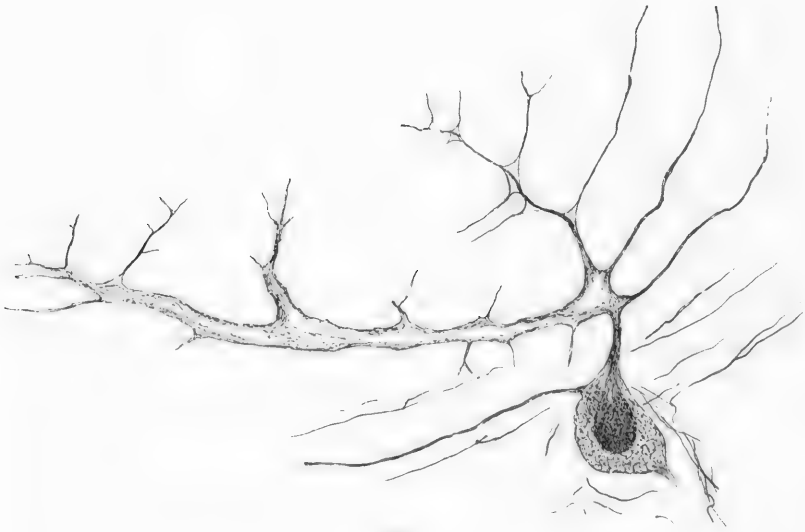


FIG. 2.

FIG. 3.



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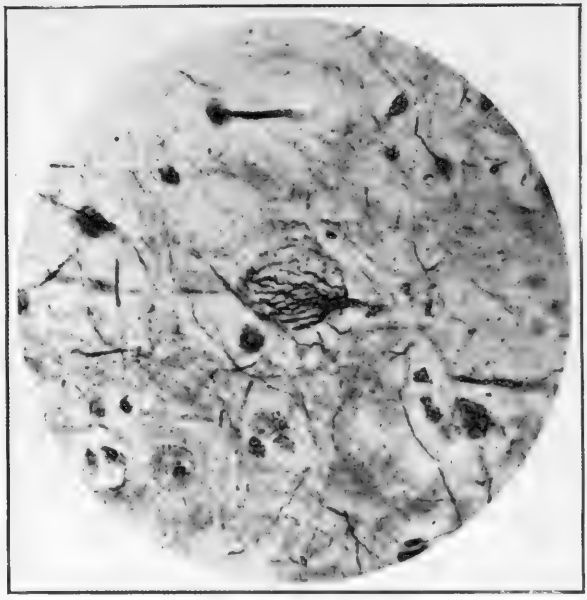

FIG. 5.

FIG. 6.

FIG. 7.

FIG. 8.

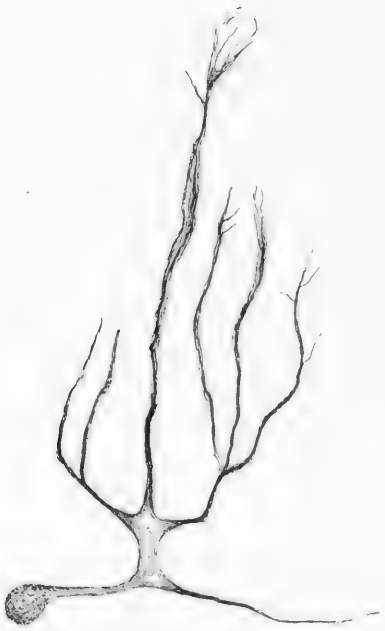

FIG. 9.

FIG. 10.

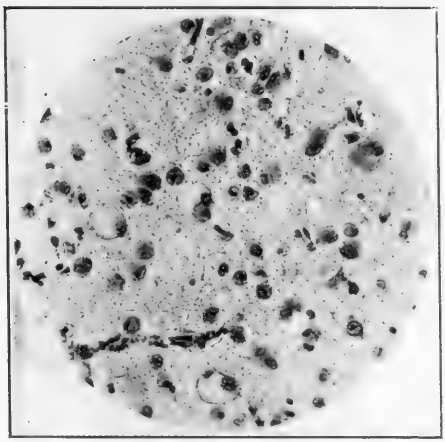

FIG. 11.

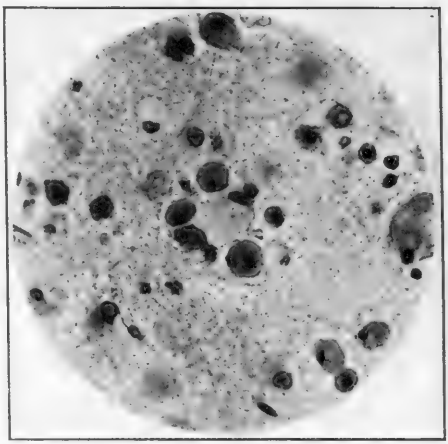

FIG. 12.

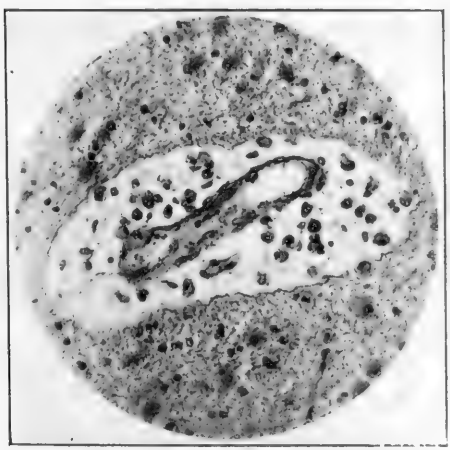

FIG. 13.

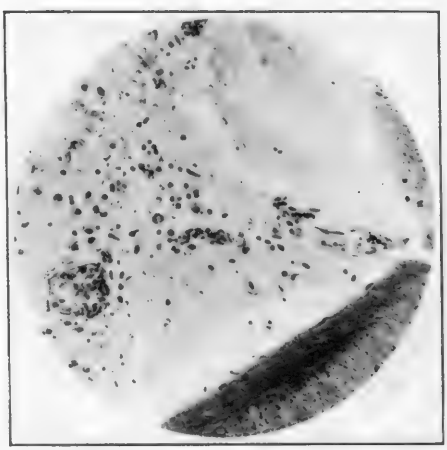

FIG. 14.

PLATE LXII.

FIG. 3. Purkinje cell showing very marked swelling of dendrite with remains of neurofibrils and their displacement. × 320.

FIG. 4. Purkinje cell showing very marked swelling of the dendrite and the remains of endocellular and perinuclear network (Bielschowsky).

PLATE LXIII.

FIG. 5. A large cortical cell showing the anastomosing epicellular network.

FIG. 6. Pyramidal cell of the cortex showing the remains of the cellular network (Bielschowsky).

FIG. 7. Same cell as Fig. 8. Different focus, showing changes in the endocellular network which are the result of degenerative changes (Bielschowsky).

PLATE LXIV.

FIG. 8. Spinal cord. Longitudinal section showing the epicellular network upon the surface of large anterior horn cell (Bielschowsky). Same cell as shown in Fig. 7.

FIG. 9. Purkinje cell showing disintegration of endocellular network, swelling of the dendrites and the peripheral position which the fibrillae assume in these swellings.

FIG. 10. Large pyramidal cell in cortex showing beginning degeneration of endocellular network. Cajal method.

PLATE LXV.

FIG. 11. Brain cortex showing degenerating ganglion cells and numerous granular cells. × 250.

FIG. 12. Granular cells in the brain cortex. The central group shows them in the rôle of phagocytes around a degenerated ganglion cell. × 500.

FIG. 13. Blood vessel in the cortex showing the granular cells in the so-called perivascular lymph space. They also can be noted in the surrounding brain substance. × 250.

FIG. 14. The pia over the cerebellum showing the presence of the granular cells. × 250.

A TRANSMISSIBLE AVIAN NEOPLASM.[1] (SARCOMA OF THE COMMON FOWL.)

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PLATES LXVI–LXVIII.

Among the many recent observations on transmissible neoplasms are several which may have greatly enlarged our knowledge of tumor behavior and certainly, for the present, have somewhat confused it. The tumors of the lower animals first studied experimentally—those of the rat and mouse—were found to conduct themselves much as do human neoplasms; and results with them rather strengthened than changed our conception of tumor-characters. But there have since been discovered a number of transmissible new growths of unusual behavior, among them a sarcoma of the dog, transmissible at coitus (Sticker, Ewing), an endemic carcinoma of fishes (Plehn, Pick, Gaylord), and a new growth of hares (von Dungern and Coca), transplantable to animals of another species. All of these in their conduct differ more or less markedly from the classical neoplasms, and whether they are to be accepted as genuine tumors is still doubtful. On the other hand, it is possible that our conception of tumor-behavior, based as it is on observations among few species, has been too narrow.

At this time then the discovery and study of transmissible tumors in new species or classes of animals has an exceptional value. And it is for such reason that a sarcoma of the chicken—the first avian tumor which has proved transplantable to other individuals—will here be dealt with in some detail.

New growths are not rare among birds, and those of the common fowl have had attention from several investigators. Fibromas, myomas, lymphomas, carcinomas and sarcomas, some of them with

[1] Received for publication June 13, 1910.

metastases, have been described. Ehrenreich,[2] and Tyzzer and Ordway[3] have made attempts at transplantation, but without success, if one except an auto-transplantation of a lymphoma accomplished by the latter authors. Ellerman and Bang[4] have shown chicken leukemia to be transmissible, and in some of their animals aleukemic lymphomata resulted from inoculation. But they have also shown, as have Hirschfeld and Jacoby,[5] that the disease is dependent on a filterable virus.

The tumor here reported was found in a barred Plymouth Rock hen of light color and pure blood. It had existed for some two months before the fowl was brought to the laboratory, becoming noticeable when the host was about fifteen months old. The sarcoma described by Ehrenreich and the myxosarcoma of Tyzzer both occurred in adult fowls.

In this hen there was present, projecting sharply from the right breast, a large, irregularly globular mass. It had developed slowly, and without apparent involvement of the health of the host. Operation was done under ether and nearly all of the growth removed. When sliced it was found to have undergone a widespread coagulation-necrosis at the center, but there was a rim of translucent, rather friable, yellowish-pink tissue of glistening, finely striated surface. Macroscopically, the growth suggested a sarcoma. Bits of it were at once inoculated by means of a trocar into the other breast and peritoneal cavity of the host. Like inoculations were also made into two hens from the same setting of eggs. Thirty-five days later the original host was dead of intraperitoneal growths, and in the breast of one of the other fowls, a large nodule had developed. A summary of the protocols follows.

Oct. 1, 1909. *Operation on Original Tumor.*—The fowl bearing the growth is a strong, young hen. The mass is situated on the right breast, in the subcutaneous tissue, and is somewhat movable. It is irregularly spherical in shape, firm, smooth, well-defined, and projects sharply from the breast contour. It

[2] M. Ehrenreich and L. Michaelis, *Zeit. f. Krebsforsch.*, 1906, iv, 586; M. Ehrenreich, *Med. Klin.*, 1907, iii, 614.

[3] E. E. Tyzzer and T. Ordway, *Jour. of Med. Research*, 1909, xxi, 459.

[4] V. Ellerman and O. Bang, *Cent. f. Bakt., Orig.*, 1908, xlvi, 595; *Zeit. f. Hygiene u. Infektionskrank.*, 1909, lxiii, 231.

[5] H. Hirschfeld and M. Jacoby, *Zeit f. klin. Med.*, 1909–10, lxix, 107.

measures 4.8 by 4.1 by 4.1 centimeters. At its outer pole the skin is attached and thinned, with several slight ulcerations over which are dry, dark-red crusts. Elsewhere, the skin is uninvolved, though the subcutaneous fat is thinned.

Under ether most of the mass was to-day excised, a piece 1 by 3 by 0.5 centimeters being left *in situ*. The growth shelled out easily from the surrounding tissue, and was enclosed in a capsule well supplied with blood-vessels.

The part removed is found on section to consist of a rim of translucent, rather friable tissue about a center that has undergone coagulation necrosis and is firm, yellow and opaque. The rim-tissue is glistening, yellowish-pink, finely striated. No debris comes away from it on scraping, and its surface remains smooth. The region of coagulation-necrosis is serpiginous in outline and forms much the larger part of the mass.

Implantation was made by means of a large trocar into the muscles of the left breast of the same fowl and also into the peritoneal cavity. Bits of the tumor-rim approximating 0.03 cubic centimeter were thrust into each site. Similar implantations were carried out on two young hens of the same brood. No cultures were taken at this time.

Nov. 5, 1909. *Autopsy of the Tumor Fowl, Which Died Yesterday.*—The fragment of the original growth is no longer to be found, but in the muscle of the left breast is an ovoid mass 1.5 by 1.5 by 2.2 centimeters, with similar necrotic center and translucent marginal zone. It has no definite capsule and is not sharply separated from the muscle about it. The peritoneal cavity contains about 20 cubic centimeters of a thin, straw-colored fluid. Attached to the lower margin of the liver, to the oblique membrane, and to the parietal peritoneum are many firm, pale yellow, ovoid or globular nodules, the largest about 1 centimeter in diameter. On section these resemble the nodule in the left breast, except that in them the necrosis is irregularly distributed. At the pelvic region, where several of the masses have coalesced, softening and necrosis are extensive. Smears from fragments taken here show no tubercle bacilli; but with aqueous methylene blue a large rod-shaped bacillus, presumably a post-mortem invader, is demonstrable. No growths are present in the other organs.

Nov. 5, 1909. In the muscle of the left breast of one of the young hens inoculated on October 1 is a firm mass measuring 2.5 by 3.5 centimeters. (The remainder of this protocol is given further on.)

Microscopic preparations of the original growth, and of the nodules developing elsewhere in the host on implantation, have shown it to be a spindle-celled sarcoma. The growths from all latter transplantations have yielded similar pictures, so the histology of the tumor will now be taken up.

In a typical section one observes loose bundles of spindle-cells coursing in every direction, and separated from the lesser blood-vessels only by endothelium (Plate LXVII, Figs. 3 and 4). Where such a bundle is cut transversely, the appearance is that of a group of round cells of varying sizes. With Mallory's phosphotungstic

acid stain intercellular fibrils are demonstrable, though they are rare in the more cellular portions of the growth. The spindle cells, while in general large, vary much in size and shape; some are short and plump, some continued into long, slender processes. The nucleus is, as a rule, large and vesicular, with a loose network and several coarse masses of chromatin. Occasionally, it is rod-shaped, and not seldom pyknotic. To the more slender cells, it gives a bulged outline at that region where it is located. Mitosis is fairly frequent and cells with two to five or six nuclei are not rare. These small giant-cells give to the growth here and there a somewhat polymorphous appearance. They are especially to be found where necrosis is beginning. The widespread necrosis seems in general dependent on insufficient vascularization, although hemorrhage from the thin-walled vessels is also occasionally responsible. In some of the later growths a myxomatous degeneration has separated the cells, and in at least one there has followed a liquefaction with formation of a cyst.

The original tumor was better encapsulated than those resulting on transplantation. Indeed, it gave clinically the picture of a benign growth until after its dissemination at operation. The apparent liberation from restraints that took place then is a phenomenon that has been noted by others[6] for the neoplasms of rats and mice. Infiltration has been the rule since. Search shows that it was also present to some extent in the original growth before operation, despite the considerable encapsulation. The tumor cells had pressed through this capsule here and there and invaded the muscle. The muscle fibers in their breaking down furnished on transverse section some remarkable pictures of pseudo-giant-cells (Plate LXVIII, Fig. 5).

Following the growth's successful transplantation, an attempt was made to propagate it further. At this writing, it is in its fourth generation. The results bring out in an interesting way the importance of blood-relationship to the transmission. The tumor has never thriven except in the intimately related fowls of the pure-blood stock in which it was first noticed. The members of this stock were few and their relationship to one another can be stated with considerable accuracy.

[6] L. Loeb, *Jour. of Med. Research*, 1901, vi, 28.

The tumor was found in one of six hens of pure blood from the same setting, and these, with a single cock of pure blood from another source, formed the parent birds of the stock. From them a single generation of chickens had been raised when the tumor was noted. For the first transplantation two of the parent hens (of the same setting as the tumor fowl) were used, but in the later ones the younger generation of chickens was employed. These, which numbered in all only between twelve and fifteen, had at least one parent in common, some of them two, and some may have been the offspring of the tumor hen, or of that in which the neoplasm grew on its first transplantation. It would be interesting to know the exact relationship between these two hens and the young fowls that proved susceptible, but that cannot be ascertained because the eggs were mixed indiscriminately for setting. Yet it is evident enough that the relationship between all of the fowls of the special stock was a very close one.

Out of twelve of this stock to which it has been transplanted, the tumor has grown in three, and they have been the hosts for the tumor generations thus far accomplished.[7] In sixteen market-bought Plymouth Rocks, superficially like the tumor stock but presumably of impure blood, no growth has been obtained; nor has it occurred in five chickens of mixed breed. In two of three market-bought Plymouth Rocks, which were less than three months old, a transient growth, followed by retrogression, was noted (Chart 1). In two pigeons and in two guinea pigs the results have been negative.

The transplantation appears to succeed better in young fowls, judging from its partial success in the young, market-bought Plymouth Rocks and its complete failure in adults of the same sort. At present, the tumor, while still growing in hosts of the special stock, is growing slowly; slow growth may be attributable to the fact that no young fowls are available, for all of the chickens of the second generation of this stock are now adult.

Only two fowls thus far have died as a result of the tumor, which attains a large size before the general health is much affected.

[7] Since this was written transplantation to similar fowls of pure blood from another source has proven successful.

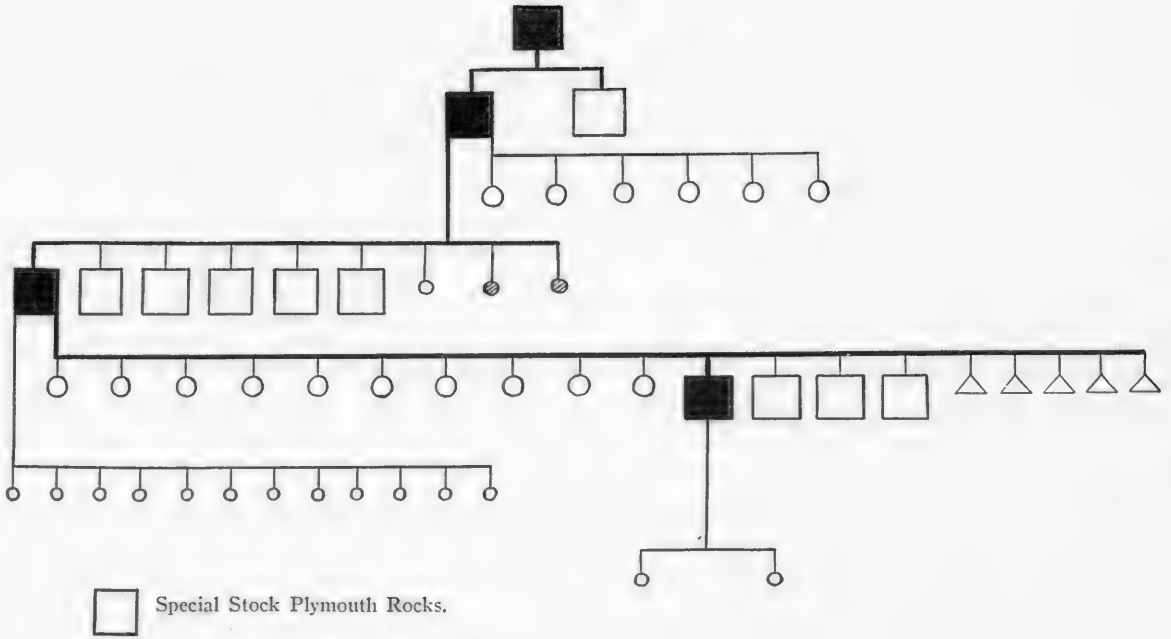
Special Stock Plymouth Rocks.
Adult, Market-bought Plymouth Rocks.
Young, Market-bought Plymouth Rocks.
Chickens of Mixed Breed.

CHART 1. Chart showing the course of transmission of the chicken sarcoma. The blackened figures represent individuals in which the tumor grew; the cross-barred ones those in which a nodule appeared but retrogressed.

(Plate LXVI, Figs. 1 and 2). Thorough bacteriological examinations of the neoplasm have twice been made at operation, but with negative results. Metastasis has occurred once, a tumor nodule being found in the left ventricle of a hen (second generation B) from which the growth had been partially removed some weeks previously (Plate LXVIII, Fig. 6). Whether the operative procedure or a natural dissemination was responsible for this metastasis cannot, of course, be determined. Many intraperitoneal inoculations, and reinoculations of negative individuals have been made, but growth has followed none of these.

A brief summary of the remaining protocols to date will now be given.

First Generation A.—Nov. 8, 1909. One of the 2 young hens inoculated with bits of the original growth was observed 3 days ago to have in the left breast a mass measuring 2.5 by 3.5 centimeters. This was operated upon today and found to be a tumor-nodule. Bits of it were transferred with the trocar to the fowls of *second generation A.*

Dec. 12, 1909. Following operation on the nodule it grew rapidly and to-day measured 5.3 by 4 by 3.5 centimeters, when it was again cut into for the sake of inoculation to *second generation B.* Hemorrhage was so profuse, and the mass was found to extend so deeply into the breast tissue that the fowl was killed. At autopsy, in addition to the mass on the breast, there was present a discrete, firm, translucent, grayish-pink nodule, 0.4 centimeter in diameter, in the substance of the left ventricle. Microscopically, this proved to be a metastasis. The other organs were normal. Fragments of the large growth were placed in bouillon and litmus milk, on sheep-serum agar, plain agar, and Loeffler's blood-serum. Also cultures were taken on gelatine, glucose bouillon, potato, the peptone medium, and glucose agar. These were under observation for several weeks. Bacterial growth occurred in none of them. The Loeffler's blood serum was quite actively digested by the fragment resting on it. Direct smears from the fresh tissue were examined for bacteria, including acid-fast bacilli, but with negative results.

May, 1910. The other animal of *first generation A* has remained free of tumor and healthy to date.

Second Generation A.—Nov. 8, 1909. Six market-bought Plymouth Rock chickens, 2 pigeons and 2 guinea-pigs were inoculated with tumor-bits in the muscle of the right breast and also intraperitoneally. The material was obtained from *first generation A* (q. v.).

Jan. 27, 1910. The chickens were reinoculated, this time into the left breast, with material from *second generation B.*

May, 1910. All are still without sign of tumor.

Second Generation B.—Dec. 10, 1909. Three market-bought Plymouth Rock chickens less than 3 months old, and 6 young fowls, 6 months old, of the tumor

stock, were inoculated in both breasts with bits of the growth from the positive fowl of *first generation A*. The 3 market-bought chickens sickened and died within 12 weeks thereafter. In one was no trace of tumor; in the second was a small nodule (0.6 centimeters in diameter) that had undergone myxomatous degeneration; in the third, a small cyst (1.0 centimeters broad), formed by degeneration and liquefaction of tumor-tissue. Some fragments of this tissue still existed at the well-encapsulated periphery of the cyst.

Jan. 17, 1910. Of the 6 fowls from the tumor stock one had developed at this date a tumor mass 1.3 centimeters in diameter. Jan. 27, 1910. The nodule measured 2.5 centimeters and was operated upon for transfer into *third generation A* (q. v.) and for reinoculation of *second generation A*. At this time many cultures were taken and stains made for bacteria but with negative results. During the next few weeks, the mass decreased in size, and no sign of renewed growth was observed until March 21, 1910. April 7, 1910. Growth has of late been rapid and the mass when today cut into measured 4 by 3 by 2 centimeters. Inoculation was done into *third Generation B*. April 18, 1910, the mass operated upon measured 5 by 3.3 by 2.5 centimeters and was approximately egg-shaped, firm and smooth (Plate LXVI, Fig. 1). The wound had healed perfectly.

In none of the other fowls of this generation was growth obtained.

Third Generation A.—Jan. 27, 1910. Five young fowls of mixed breed, 10 market-bought Plymouth Rocks, and 4 chickens, seven months old, of the tumor-stock were inoculated from *second generation B*.

May 9, 1910. To date none have shown tumors except a cock of the tumor-stock in which was noted March 21 a small lump on the left breast. Operation was performed April 7, and inoculation made into *fourth generation A*.

Third Generation B.—April 7, 1910. Twelve market-bought Plymouth Rocks, under 3 months of age, were inoculated in the muscle of both breasts and in the subcutaneous tissue of the left breast with material from *second generation B*. June 1, 1910. In none of these has growth appeared.

Fourth Generation A.—April 7, 1910. Two of the young fowls inoculated on this date received in the right breast material from *third generation A* instead of from *second generation B*. June 1, 1910. Both of these are negative as regards tumor.

So far as tested this new growth in the chicken has proved itself a neoplasm of classical behavior. The peculiarities which it exhibits are those already made familiar through observations on the tumors of the rat, mouse, dog and man. The tissue specificity which has limited its successful transplantation to fowls of the stock in which the primary growth arose is striking, but not more so than the specificity of certain mouse and rat tumors; and this character may in part explain why previous attempts to transplant neoplasms of the fowl have failed.

The tumor is at best so difficult of propagation that no attempts have been made to determine whether it can be transmitted by cell-

fragments, or by cell-free derivatives. For the same reason, the question whether growth takes place entirely from the introduced cells has not been investigated. But there is no reason to suspect that the neoplasm will differ on these points from the better-known tumors of mammals.

It may not be superfluous to point out that such similarity of behavior as has been thus far observed between this avian tumor and those of mammals was, after all, largely to be expected. A graft of mammalian tumor succeeds better in a blood-related individual, and a young one, not because it is tumor, but because it is tissue. The tissue laws here concerned are probably not very different in birds. Nevertheless, the close correspondence in behavior between this avian tumor and the typical mammalian tumors is certainly of interest. Sticker's lymphosarcoma of the dog, which is transplantable to foxes, von Dungern's tumor of the hare, which will also grow in rabbits, both deviate more from the tumor-type as observed in mammals than does this sarcoma of the fowl. At first sight, indeed, the behavior of these unusual growths seems in absolute violation of the laws governing tissues. Yet this is not necessarily true. For in the hybridization of the horse with the ass, of the dog with the wolf, the elements from different species unite in a much more intimate association than exists between a tumor and its host.

SUMMARY.

In this paper is reported the first avian tumor that has proved transplantable to other individuals. It is a spindle-celled sarcoma of the hen, which thus far has been propagated into its fourth tumor generation. This was accomplished by the use of fowls of pure blood from the small, intimately related stock in which the growth occurred. Market-bought fowls of similar variety have shown themselves insusceptible, as have fowls of mixed breed, pigeons and guinea-pigs. The percentage of successful transplantations has been small, but in the individuals developing a tumor its growth has been fairly rapid. Young chickens are more susceptible than adults. The reinoculation of negative fowls has never resulted in a growth.

Throughout, the sarcoma has remained true to type. It is

infiltrative and destructive. Metastasis has been observed once (to the heart). Experiments to determine whether the growth may be transmitted by cell-fragments have not yet been made. Repeated bacteriological examinations have yielded negative results.

In its general behavior, so far as tested, this avian tumor closely resembles the typical mammalian neoplasms that are transplantable.

EXPLANATION OF PLATES.

PLATE LXVI.

FIG. 1. Sarcoma. Second generation B.

FIG. 2. Cross-section of same tumor, somewhat enlarged.

PLATE LXVII.

FIG. 3. Sarcoma of the chicken from an intraperitoneal growth.

FIG. 4. Sarcoma of the chicken.

PLATE LXVIII.

FIG. 5. Invasion of muscle by the sarcoma.

FIG. 6. A metastasis in the heart wall. The lumen shown at the left hand corner is that of a small vein.

FIG. 1.

FIG. 2.

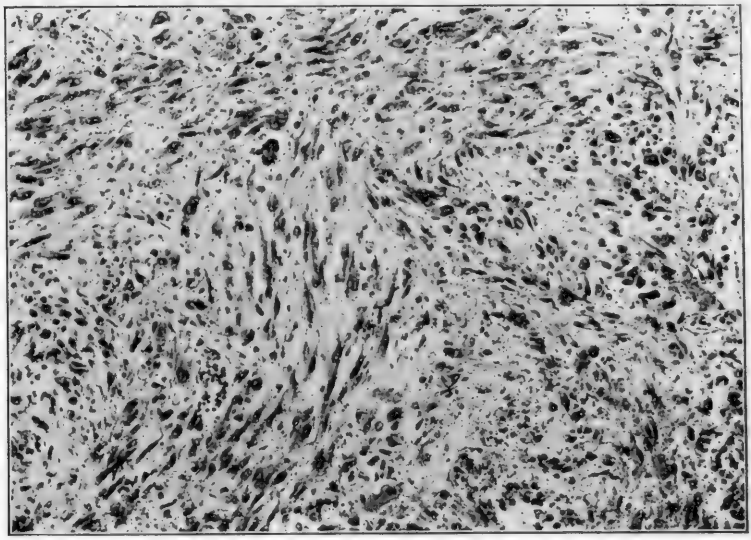

FIG. 3.

FIG. 4.

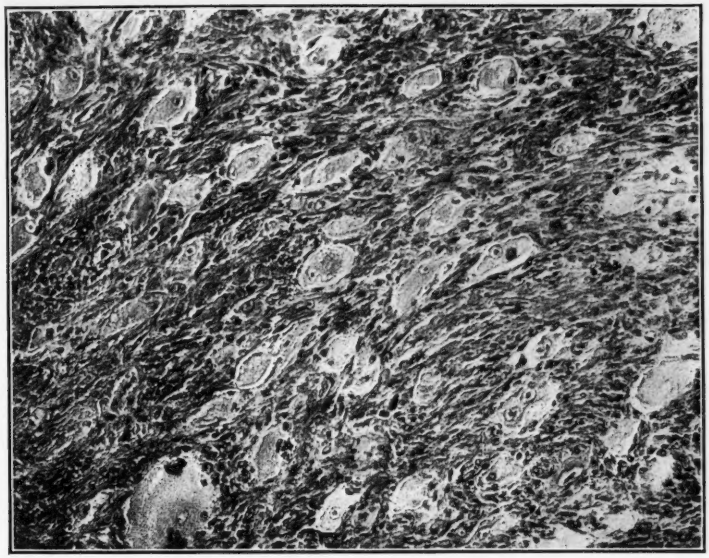

FIG. 5.

FIG. 6.